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BIO-INGENIEURSWETENSCHAPPEN

UNCOVERING THE PRIMING POTENTIAL OF THE GREEN LEAF VOLATILE Z-3-HEXENYL ACETATE

TOWARDS A NEW DISEASE CONTROL TOOL

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Cover photo: View of the wheat field on the experimental farm in Bottelare.

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Abbreviations

13-HP	13-hydroperoxide fatty acid
AAT	alcohol acyltransferase
ABA	abscicic acid
ADH	alcohol dehydrogenase
ADR	aldehyde reductase
AKR	aldo-keto reductase
ALA	α-linolenic acid
ANOVA	analysis of variance
AOS	allene oxide synthase
AzA	azelaic acid
BABA	β-aminobutyric acid
BIR	Beneficial microbe induced resistance
BR	brassinosteroid
BTH	benzo-(1,2,3)-thiadiazole-7-carbothiolic acid S-methyl ester
BVOC	biogenic volatile organic compound
CAD1	cinnamyl alcohol dehydrogenase 1
CaM	calmodulin
CCR3	cinnamoyl CoA reductase 3
CK	cytokinin
CnC	Cap'n'collar protein
COI1	coronatine insensitive 1
COR	coronatine
DA	dehydroabietinal
DACC	depolarization-activated calcium channel
DAMP	damage associated molecular patterns
DIMBOA	2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one
DIR1	DEFECTIVE IN INDUCED RESISTANCE1
DON	deoxynivalenol
E-2-HAC	E-2-hexenyl acetate
E-2-HAL	E-2-hexenal
E-2-HOL	E-2-hexenol
ET	ethylene
ETI	effector triggered immunity
ETS	effector triggered susceptibility
FA	fatty acids
FAC	fatty acid - amino acid conjugate
FHB	Fusarium head blight
FMO1	FLAVIN-DEPENDENT MONOOXYGENASE 1
G3P	glycerol-3-phosphate
GA	giberellin/giberellic acid
GABA	γ-aminobutyric acid

GAD	glutamate decarboxylase
GFP	green fluorescent protein
GLA1	glycerolipase A1
GLR	GLUTAMATE RECEPTOR-LIKE
GLV	green leaf volatile
GOGAT	glutamine:2-oxoglutarate aminotransferase
GS	glutamine synthetase
GT	glycosyltransferase
HAI	Hours after inoculation
HexVic	(Z)-3-hexenyl-O-α-L-arabinopyranosyl-(1,6)-β-D-glucopyranoside
HI	hexenal isomerase ((3*Z*):(2*E*)-enal isomerase)
HIPV	herbivore induced plant volatile
HIR	herbivore induced resistance
HPL	hydroperoxide lyase
HR	hypersensitive response
HST	host-selective toxins
IAA	indole-3-acetic acid
ICS	isochorismate synthase
INA	2,6-dichloroisonicotinic acid
IP	invasion pattern
IPM	integrated pest management
IPR	invasion pattern receptors
IPRT	IP triggered response
ISR	induced systemic resistance
JA	jasmonic acid/jasmonate
JAZ	JASMONATE–ZIM DOMAIN
JOX	JASMONATE-INDUCED OXYGENASE
LOX	lipoxygenase
MAMP	microbial-associated molecular patterns
MeJA	methyl jasmonate
MPK	mitogen-activated protein kinase
MPKK	MPK kinase
MPKKK	MPK kinase kinase
NADPHox	NADPH oxidase
NPR1	NONEXPRESSOR OF PR GENES 1
OPLS-DA	orthogonal partial least squares discriminant analysis
OS	(insect) oral secretions
PA	polyamine
PAL	phenylalanine ammonia lyase
PCA	principal component analysis
PCD	programmed cell death
PDA	potato dextrose agar
PEROX	peroxidase

PGPF	plant growth promoting fungi
PGPR	plant growth promoting rhizobacteria
Phe	L-phenylalanine
PI	proteinase inhibitor
PIR	Pathogen induced resistance
PIP	Pipecolic acid
PLC	Programmable logic controller
PPP	plant protection product
PR	pathogenesis related
PRR	pattern recognition receptor
PTI	pattern-triggered immunity
RES	reactive electrophilic species
ROS	reactive oxygen species
RT-qPCR	Reverse transcription polymerase chain reaction
SA	salicylic acid/salicylate
SAR	systemic acquired resistance
SUS	shared and unique structure
TCA	tricarboxylic acid
TE	treatment effect
TF	transcription factor
TI	Bowman-Birk type trypsin inhibitor
VIP	variable in importance projection
VIR	Volatile induced resistance
V_m	plasma membrane potential
WT	Wild type
Z-3-HAC	Z-3-hexenyl acetate
Z-3-HAL	Z-3-hexenal
Z-3-HOL	Z-3-hexenol

Chapter 1 Problem Statement and Outline

1.1 Crops under pressure and the need for innovative crop protection tools

To optimize crop yield and quality, farmers have relied on the use of fertilizers and agrochemicals to provide optimal nutrient concentrations for the crop and to realize appropriate protection against insect pests and pathogens. However, in the arms race against pathogens and pests, yield is under constant pressure of pathogens adapting to the control measures and agro-ecosystem in which they live. Plant diseases and pests can have devastating impacts and globally constitute yield losses up to 20% (Oerke, 2006). In addition, climate change is an increasingly important driver in the spread of pathogens and pests which poses new challenges for disease management in the future (Miraglia *et al.*, 2009; Bebber *et al.*, 2013; Bebber *et al.*, 2014a; Váry *et al.*, 2015; Battilani *et al.*, 2016). To gain a better insight in the global pest and pathogen distribution Bebber *et al.* (2014b) made a distribution model taking into account both biogeographical and socio-economic factors. Intriguingly, they found that with increasing gross domestic product per capita (**GDP**), reported pest numbers increase. However, as GDP is most likely linked to observational capacity, this means that in less developed countries a vast number of crop pests (including plant pathogens, nematodes, insect herbivores) remain undetected, implying that plant disease occurrence currently is greatly underestimated (Figure 1-1). In the view of increasing and emerging crop diseases, an adequate crop protection system therefore is vital to safeguard current and future food quantity, -quality and -safety (Bebber & Gurr, 2015).

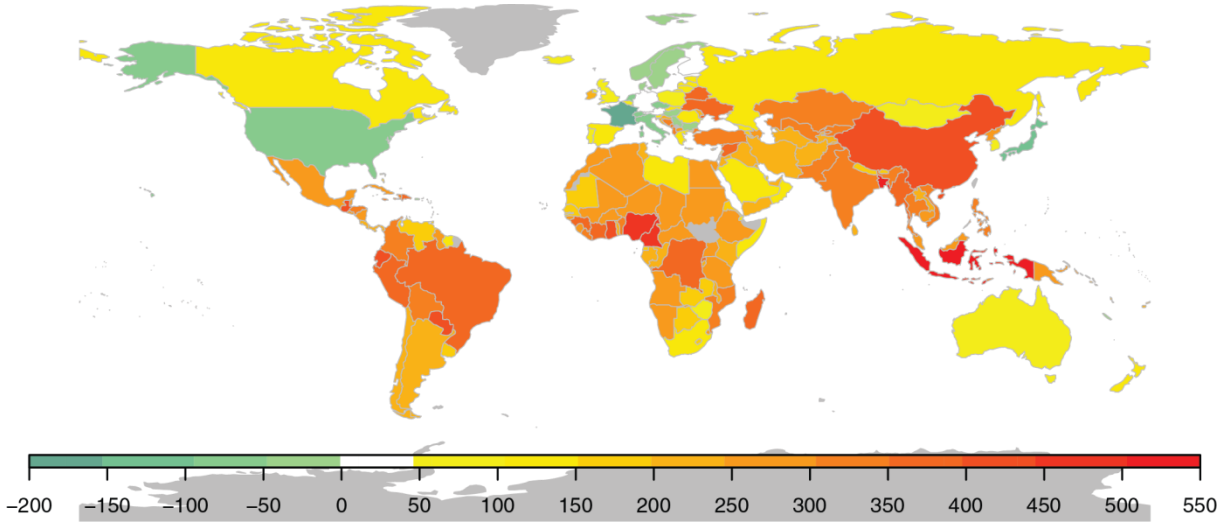

Figure 1-1 Expected additional numbers of pests per country if the per capita gross domestic product (GDP) and investment in research and development (R&D) in each country were set to current USA levels to account for economic differences. Currently, in less-developed countries plant diseases remain undetected and as a consequence the spread of plant diseases is probably greatly underestimated (Bebber *et al.*, 2014b)

To control diseases and insect pests, biocide application has since long become commonplace and indispensable in agronomy. However, the environmental and health concerns associated with pesticide use (Lamichhane *et al.*, 2016) prompted the European Union to develop a new legislation which resulted in the EU directive on sustainable use of pesticides (2009/128/EC). This directive aims at reducing the use and risks of pesticides. To achieve a pesticide reduction, while still minimizing crop losses due to insect pests and diseases, Integrated Pest Management (**IPM**) is encouraged. Within the framework of IPM, research on novel plant protection products and biological control agents is a proposed strategy. Biocontrol has generally been defined as the practice of introducing natural enemies, antagonists or competitors, and other self-replicating biotic entities in the framework of pest management. However, others adopt a broader definition which includes enhancing natural intrinsic plant defense and the use of bio-rational pesticides (McSpadden Gardener & Fravel, 2002; Droby *et al.*, 2009). In this thesis we will use the broader definition of biocontrol. Biological control agents against deleterious insects include entomopathogenic fungi, bacteria and nematodes (Gaugler & Kaya, 1990; Kaya & Gaugler, 1993; Shah & Pell, 2003) , plant essential oils (Tripathi *et al.*, 2009), insect pheromones for mating disruption and trapping (Stenberg *et al.*, 2015), and the introduction of natural enemies against insect pests (Snyder & Ives, 2003), amongst others (Copping & Menn, 2000). Also several methods for the biocontrol of fungi have been reported such as the use of fungal and bacterial antagonists (Kiss, 2003; Chung *et al.*, 2005), metabolites of endophytic fungi (Kumar & Kaushik, 2012) and plant essential oils (Daferera *et al.*, 2003). Aforementioned agents act directly against harmful insects or pathogens; an alternative approach relies on the innate immunity of plants and aims to activate/enhance these defenses to combat invading pathogens and insect pests.

Root colonization by beneficial bacteria can induce systemic resistance (See section 2.3 for more details) against different pests, also microbe associated molecular patterns (**MAMPs**) can be applied which are recognized by the plant and induce plant defense activation. For example, chitin is a component of the cell walls of fungi and insects and has been shown to promote resistance against fungal and bacterial infections, and insect infestation (El Hadrami *et al.*, 2010). Also mimics of plant defense hormones can be used to enhance defense. Such compounds have already been commercialized under the trade names Bion® and Actigard® (Syngenta, Basel, Switzerland) which contain benzo-(1,2,3)-thiadiazole-7-carbothiolic acid S-methyl ester (**BTH**), a functional analogue of the plant defense hormone salicylic acid **(SA)** as active ingredient. Another group of natural plant compounds are green leaf volatiles (**GLVs**) which are produced after (a)biotic stress and have also been found to activate and prime plant defenses (Scala *et al*., 2013a) and thus may be considered as a promising biocontrol agent. Primed plants display either faster, stronger, or both activation of the

various cellular defense responses that are induced following attack by pathogens or insects (Conrath et al., 2006). However, while GLV production by plants has been widely known, research on GLVs in the scope of plant protection against pathogens is currently limited.
This PhD thesis aims to fill this knowledge gap and aims to acquire a better understanding on the use of GLVs in the defense of plants against pathogens.

1.2 Thesis outline

As GLVs are produced upon biotic stress and as the priming effect has already been shown in the defense against deleterious insects, we hypothesize that the **GLV Z-3-hexenyl acetate (Z-3-HAC) also primes plant defense against fungal pathogens.**
Priming by GLVs has often been associated with jasmonate (JA) dependent signaling in monocotyledonous plant species, which lets us formulate our second hypothesis: **Z-3-HAC primes for enhanced JA dependent signaling in wheat.**
As defense signaling consists of several antagonistic pathways, priming by GLVs may entail both positive and negative consequences against pathogens with a different lifestyle, leading to our third hypothesis: **priming by Z-3-HAC may prime for enhanced resistance or enhanced susceptibility, depending on the type of pathogen.**
In order to test these hypotheses, following research questions need to be answered in this doctoral thesis:

- Does priming by Z-3-HAC take place in wheat?
- Which defense pathways are associated with the priming process by Z-3-HAC?
- Which metabolites are implicated in the priming response by Z-3-HAC?
- Is Z-3-HAC a broad spectrum priming agent in wheat and rice against other pathogens?

These research questions will be answered throughout the PhD manuscript. Each chapter addresses one of the above mentioned research questions.
The introductory chapter gives an overview of current knowledge on plant-pathogen interactions (**Chapter 2**). Additionally, the concept of defense priming is highlighted.
We review the state of the art on knowledge on GLVs and investigate their role in the interaction between plants and their direct environment, within the framework of a meta-analysis (**Chapter 3**). The meta-analysis revealed that fungal infection more strongly induces GLV production compared to insect herbivore damage or mere wounding. Additionally, we found that GLV production differs between monocotyledonous and eudicotyledonous plant species upon biotic stress.

In **Chapter 4** we investigated the potential of priming by Z-3-HAC in the wheat (*Triticum aestivum* L.) - *Fusarium graminearum* model system. Several infection assays were used and the underlying defense mechanisms were investigated using bio-assays. These assays revealed that Z-3-HAC primes for enhanced defense by augmenting JA related defense pathways.

Next, we aimed to further uncover the defense signaling pathway after priming by Z-3-HAC in the interaction between *F. graminearum* and wheat (**Chapter 5**). We performed a targeted and an untargeted metabolomics study to identify important metabolites for both primed and control seedlings at different time points. Primed seedlings exhibited a strong increase in glycosylated compounds, and we found evidence of increased N transport away from the infected tissue.

Lastly, we widened our scope and explored whether Z-3-HAC can be used as a priming agent in other wheat-pathogen interactions and in another monocotyledonous crop: rice (*Oryza sativa* L.)(**Chapter 6**). These results show that priming by Z-3-HAC induced enhanced resistance for several pathogens, while increasing susceptibility to other pathogens.

Chapter 7 summarizes our main findings and places these in a wider context of plant protection. This chapter also addresses future research challenges.

Chapter 2 General Introduction

2.1 Growth versus Defense: A balancing act

Plants are continuously challenged by pathogens and insects. To survive, plants have developed different strategies to try to evade or counteract damage inflicted by these pathogens or insects. To achieve this, a timely recognition and mounting of defenses is mandatory. Jones & Dangl (2006) proposed their famous zigzag model which encompasses two branches of the plant immune system. Namely, first, the recognition of conserved microbe-associated-molecular-patterns (**MAMP**) and subsequent pattern-triggered immunity (**PTI**); and secondly, the recognition and response to virulence factors, which aim to suppress PTI and is called effector-triggered-immunity (**ETI**) (see Figure Box 1).

During their lifetime, plants are subjected to various biotic attacks such as insects, bacteria and fungi. To cope with these biotic stresses a timely recognition is mandatory for employing their defense mechanism. Plants possess membrane bound pattern recognition receptors (**PRRs**). These PRRs can recognize microbial-associated molecular patterns (**MAMPs**) after which an intricate defense network is activated. The different stages of plant immune response can be illustrated as a zigzag model (Figure Box 1) (Jones & Dangl, 2006). In short, MAMPs are broadly conserved molecules which are associated with pathogens such as chitin (component of fungal cell walls), flagellin and lipopolysaccharides which betray bacterial presence. In the first phase, perception of MAMPs leads to pattern-triggered immunity (**PTI**), which constitutes a complex signaling network leading to several defense mechanisms such as stomatal closure, generation of reactive oxygen species (**ROS**), production of antimicrobial compounds and pathogenesis related (**PR**) proteins. However, in the second phase successful pathogens employ several mechanisms to counteract PTI (e.g. altering the host cells metabolism or hijacking the plant hormone network), which leads to effector triggered susceptibility (**ETS**). These effectors can, in turn, be recognized by the plant in the third phase leading to effector triggered immunity (**ETI**), which is an accelerated and amplified version of PTI, resulting in disease resistance and often culminates in hypersensitive response (**HR**), resulting in apoptosis. Recognition of these effectors (avirulence **Avr** proteins) is accomplished by resistance (**R**) genes (Jones & Dangl, 2006; Bigeard *et al.*, 2015). As both pathogen and plant strive to gain the upper hand, this leads to a continuous arms race in which the pathogens try to evade recognition by the plants PRRs and R genes by altering their effectors, while plants are under selective pressure to adapt their immune system to these evolving pathogens (Boller & He, 2009).

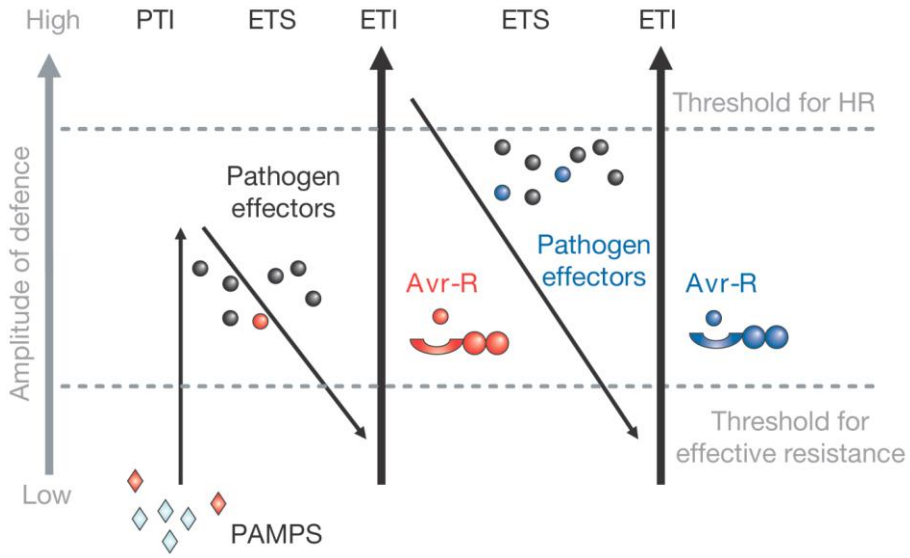

Figure Box 1: Zig-Zag Model according to Jones and Dangl (2006)

While the zigzag model nicely conceptualizes the general plant immunity response, it has (as each model), its limitations. The model is difficult to apply to necrotrophs and insects, it also does not take into account endogenous damage associated molecular patterns (**DAMPs**), which can also influence the plant response, and it does not integrate previous events in which the plant was already attacked by pathogens or insects and thus may have acquired an enhanced defense response (Cook *et al.*, 2015). Pritchard & Birch (2014) argue that in the omics era, we should not rely on one model, but instead use a range of dynamic models to encompass the different plant-pathogen systems. Cook *et al.* (2015) propose an alternative to the zigzag model, which omits the strict division between MAMPS and effectors, and replaces this with invasion patterns (**IPs**), which are perceived by IP receptors (**IPR**), leading to IP-triggered responses (**IPTR**)(Figure 2-1).

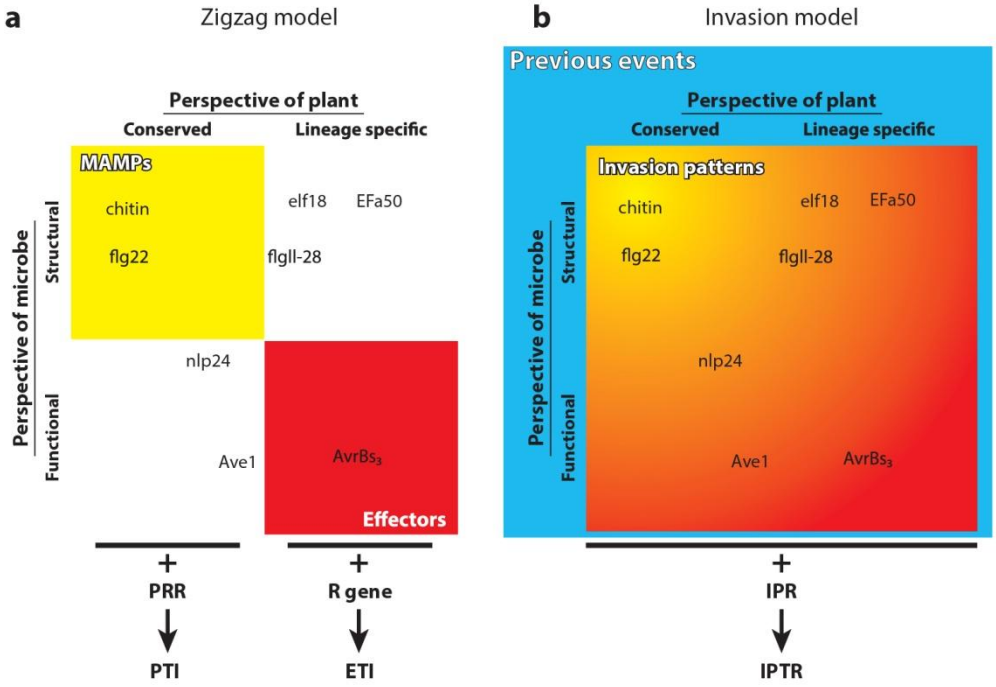

Figure 2-1 The invasion model constitutes an alternative for the zigzag model of Jones & Dangl (2006). While the zigzag model maintains a division between microbe associated molecular patterns (MAMPs) and effectors, the invasion model of Cook *et al.* (2015) places those compounds in a continuum, wherein each compound can induce plant defense. PRR: pattern recognition receptor; PTI: pattern-triggered immunity; R gene: resistance gene; IPR: invasion pattern receptor; IPTR: invasion pattern triggered response; flg22, elf18, EFa50, flgII-28, nlp24, Ave1 and AvrBs3 are examples of microbial molecules which serve as invasion patterns in plant immunity.

Regardless of the immunity model, infected plants need to activate their defenses and consequentially, resources must be allocated to the affected tissue. However, because these resources are limited, an important trade-off exists between growth and defense at the advent of biotic stress. It has generally been accepted that inducible defenses are preferable to constitutive defenses as the costly production of defensive compounds only occurs in the advent of biotic stress (Huot *et al.*, 2014).

The costs and benefits of inducing plant defense are illustrated by Baldwin (1998). He found that the activation of defense in *Nicotiana attenuata* by treating the roots with MeJA negatively affected seed production by 26%. However, in fields with high insect herbivore pressure, the induced plants survived longer and produced more seed than their non-induced counterparts. This illustrates that under certain circumstances the benefits may outweigh the costs. While allocation costs might not affect the yield in a intensively managed field, they might affect productivity in less favorable growing conditions (Heil & Baldwin, 2002).

2.2 Plant hormones play a crucial role in plant defense

2.2.1 Linking growth and defense

Plant hormones play a crucial role in the development, growth and reproduction of the plant (organs). Besides these, plant hormones have also been shown to regulate plant defense responses. The three archetypal plant defense hormones in reaction to biotic stress are SA and JA and to a lesser extent ethylene (**ET**). It has generally been assumed that defense against biotrophic pathogens is regulated by SA dependent signaling, whereas defense against necrotrophic pathogens is regulated by JA/ET dependent signaling, between which an antagonistic relationship exists (Glazebrook, 2005; Caarls *et al.*, 2015). However, later research showed this antagonism to be concentration dependent as at low concentrations of SA and JA, their respective responses work synergistically (Mur *et al.*, 2006). As knowledge on plant defense grew, it became apparent that also other growth hormones such as auxins (Kazan & Manners, 2009), cytokinins (**CK**) (Choi *et al.*, 2011), gibberellins (**GA**) (De Bruyne *et al.*, 2014), abscicic acid (**ABA**) (Asselbergh *et al.*, 2008), strigolactones (Stes *et al.*, 2015) and brassinosteroids (**BR**) (De Bruyne *et al.*, 2014) are active in plant defense. Thus, instead of a bimodal system, plant defense is modulated by an intricate network of plant hormones, which additionally exert negative or positive influences on each other. Many excellent reviews have been published on the modes of actions and signaling pathways of aforementioned hormones and we kindly refer to these to gain a more profound comprehension on plant defense signaling (Robert-Seilaniantz *et al.*, 2011; Pieterse *et al.*, 2012).

2.2.2 Plant hormones in defense: from dicots to monocots

Most research on the role of plant defense hormones has been done on the model plant *Arabidopsis*. However, *Arabidopsis* based knowledge on plant defense hormones is not

always transferable to other (monocotyledonous) species. The disparity between the model plants *Arabidopsis* and *Oryza sativa* has been reviewed in De Vleesschauwer *et al.* (2013) and De Vleesschauwer *et al.* (2014). They reported that the model in which SA induces resistance against biotrophs and JA against necrotrophic pathogens holds up for *Arabidopsis*, while in rice JA has also been shown to induce resistance against a broad range of pathogens with varying lifestyles and infection strategies such as the (hemi)biotrophic pathogens *Xanthomonas oryzae* and *Magnaporthe oryzae* thereby challenging the dichotomous model of *Arabidopsis* (Figure 2-2). Interestingly, this contrast between *Arabidopsis* and rice also holds up for wheat, another monocot crop. For example, *Blumeria graminis* is an obligate biotrophic pathogenic fungus on wheat against which resistance is regulated in a gene for gene relationship. One would expect that defense against this fungus is regulated by SA inducing a HR response. However, several studies have shown that JA confers resistance against *B. graminis* by upregulating PR genes and altering its polyamine biosynthesis (Walters *et al.*, 2002; Duan *et al.*, 2014).

Another main difference between *Arabidopsis* and rice lies within the SA signaling. In *Arabidopsis* almost all BTH responsive genes are NONEXPRESSOR OF PR GENES 1 (**NPR1**) dependent, while in rice BTH-upregulated genes branch off in an OsNPR1 and OsWRKY45 dependent pathways. Furthermore, De Vleesschauwer *et al.* (2014) suggest a scenario whereby OsNPR1 acts as an energy switch enabling plant resources to be diverted to the OsWRKY45-dependent pathogen defense.

These cases illustrate that the *Arabidopsis* model cannot be completely transferred to monocotyledonous crops and that several signaling pathways contribute to plant defense, which is mainly dependent on the pathogen type and its infection strategy. In addition, several plant pathogens are known to produce phytotoxins and are able to hijack the defense hormone network, which further complicates the defense signaling. Hence, a deep understanding of the involved defense signaling is needed in order to find novel methods to combat plant pathogens.

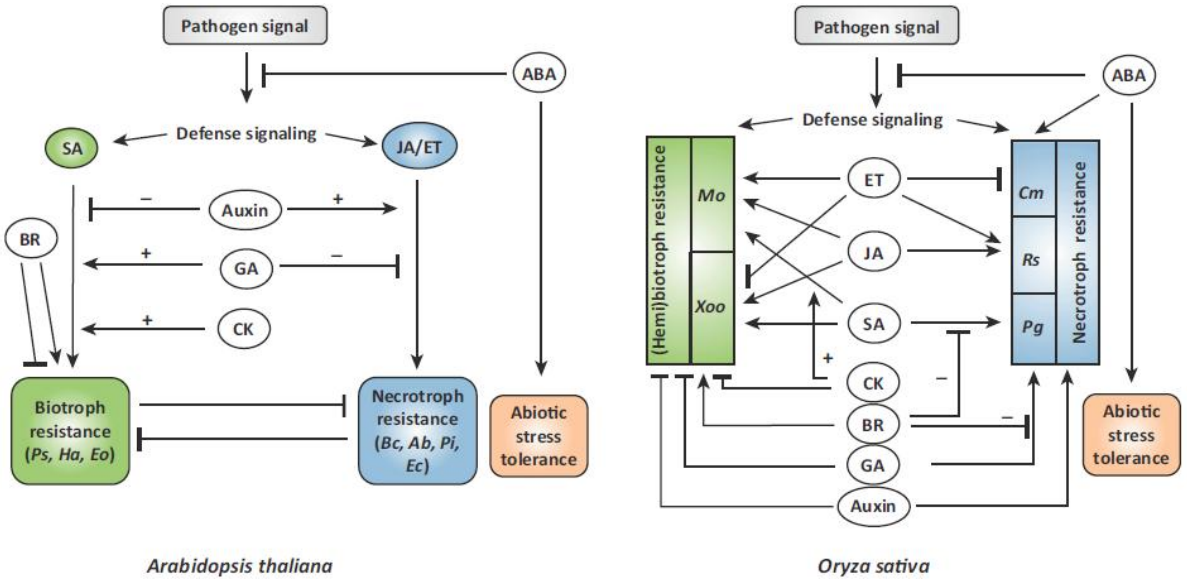

Figure 2-2: Diagram depicting the hormone network and synergistic/antagonistic signaling for the model plants *Arabidopsis* (left) and *Oryza sativa* (right). Arrows represent positive interactions between hormone signalling pathways, while blunt-ended arrows depict antagonistic interactions between different hormone signalling pathways. Plant hormone abbreviations: SA, salicylic acid; JA, jasmonic acid; ET, ethylene; BR, brassinosteroids; GA, giberellic acid; CK, cytokinins; ABA, abscicic acid. Pathogen abbreviations: Ab, *Alternaria brassicicola*; Bc, *Botrytis cinerea*; Cm, *Cochliobolus miyabeanus*; Ec, *Erwinia carotovora*; Eo, *Erysiphe orontii*; Ha, *Hyaloperonospora arabidopsidis*; Mo, *Magnaporthe oryzae*; Pg, *Pythium graminicola*; Pi, *Pythium irregulare*; Ps, *Pseudomonas syringae*; Rs, *Rhizoctonia solani*; Xoo, *Xanthomonas oryzae pv. oryzae* (De Vleesschauwer *et al.*, 2013)

2.2.3 Hijacking the plant hormone network

The spatio-temporal aspects of plant-pathogen interactions are of paramount importance. To limit losses against invading pathogens or insect herbivores, plants need to regulate their defense in a timely manner. Because of the many antagonistic defense signaling pathways, a wrongly timed activation of a signaling pathway may counteract other signaling pathways, making plants more vulnerable for attack. This antagonistic signaling has also been exploited by plant pathogens through co-evolution (Maor & Shirasu, 2005; Pieterse *et al.*, 2009). By producing (analogues of) phytohormones or inducing certain signaling pathways, pathogens can hijack the plant defense network, making them more susceptible to infection (Yan & Xie, 2015). The classical example is the production of the phytotoxin coronatine (**COR**), a mimic of JA-isoleucine, by the bacterium *Pseudomonas syringae.* Plant defense against infection by *P. syringae* in *Arabidopsis* is mediated by SA. However, by releasing COR, the JA defense pathway is activated which suppresses SA defense, leading to reduced resistance. Fungi can also tap in to the defense regulatory network and induce susceptibility. Known examples are the production of plant hormone ethylene by *Cochliobolus miyabeanus* which antagonizes ABA mediated defense in rice (De Vleesschauwer *et al.*, 2010; De Bruyne, 2015), gibberellic acid for *Fusarium fujikuroi* (Wiemann *et al.*, 2013) and ABA by *Botrytis cinerea*, which antagonizes SA dependent defense responses (Kettner & Dörffling, 1995; Audenaert *et al.*, 2002a).

We can conclude that the outcome of a plant-pathogen interaction is determined by the level on recognition of the pathogen by the plant and by the intricate interplay of plant defense and interference of that defense by the invading pathogen.

2.3 SAR, HIR, ISR and priming, different concepts or variations on the same theme?

2.3.1 Systemic resistance

After plants are attacked by pathogens or insects, the infected/infested tissue will mount its defenses. Concurrently, defenses can also be upregulated in tissue distal from the site where the initial defense trigger occurred. This upregulation of distant defenses will promote protection against a broad range of pathogens/insect herbivores. This systemic resistance has generally been divided in systemic acquired resistance (**SAR**) and induced systemic resistance (**ISR**). While both mechanisms confer induced resistance in distal tissue which has not yet been attacked, SAR has generally been associated with systemic resistance after a pathogen attack, whereas ISR is associated with systemic resistance after root colonization with beneficial microbes (Figure 2-3).

Following pathogen attack, the transcription cofactor NPR1 is needed to initiate defense genes that contribute to SAR. NPR1 occurs as an oligomer in the cytosol and its concentration is regulated by the SA receptors NPR3 and NPR4. After PTI or ETI (Figure Box 1), SA accumulates and induces a redox change in the cell which facilitates monomerization of NPR1 after which NPR1 is translocated to the nucleus where it interacts with the TGA family[1] of transcription factors, which together with WRKY transcription factors activate SA responsive defense genes. These include the upregulation of PR genes which encode proteins with different antimicrobial activities (e.g. chitinases, β-1,3-glucanase, thaumatin like proteins) (Durrant & Dong, 2004; Fu & Dong, 2013).

The mobile signal by which SAR is activated in distal tissue is still unknown, MeSA, ROS, dehydroabietinal (**DA**), lipid derived signaling molecules, glycerol-3-phosphate (**G3P**)-dependent factor, azelaic acid (**AzA**), pipecolic acid and peptides have been suggested (Fu & Dong, 2013; Pieterse *et al.*, 2014; Shah & Zeier, 2014). A study investigating the mobile signal of SAR showed that in *Arabidopsis* the lipid-transfer protein DEFECTIVE IN INDUCED RESISTANCE1 (**DIR1**) is indispensable for G3P, DA and AzA induced activation of SAR and is likely to act as a chaperone for the mobile signal (Maldonado *et al.*, 2002; Shah & Zeier, 2014). As several compounds have been shown to induce SAR, this suggests that the

[1] The TGA family of transcription factors binds with the sequence 5'-TGA(G/C)TCA-3'

mobile SAR signal depends on the type of attacker or on the defense pathway that is locally activated.

In distal tissue, SA accumulation has been shown to be critical for SAR establishment. Using mutant plants which are not able to accumulate free SA it was found that these plants did not activate SAR (Vernooij *et al.*, 1994). However, 2,6-dichloroisonicotinic acid (**INA**) and BTH, two SA analogs, were able to induce SAR without the accumulation of SA, suggesting that SAR can be induced independently or downstream of SA signaling (Delaney *et al.*, 1995; Friedrich *et al.*, 1996). Furthermore, the protein FLAVIN-DEPENDENT MONOOXYGENASE 1 (**FMO1**) is required in systemic tissue to amplify and transduce the SAR signal from the primary infected leaves (Mishina & Zeier, 2006).

However, SAR responses do not solely involve SA dependent pathways and they can also influence JA biosynthesis. Truman *et al.* (2007) showed in *Arabidopsis* that after infection with an avirulent strain of *Pseudomonas syringae*, a SAR transcriptional response was activated in systemic leaves. Concomitantly, in phloem exudates of challenged leaves an increase in the plant hormone JA was present, coinciding with an increased transcription of JA biosynthesis genes and an accumulation of JA in systemically responding leaves. In contrast, Attaran *et al.* (2009) found that SAR was still maintained in JA insensitive mutants which argues that JA is not an exclusive SAR mobile signal and that more research is needed on the role of JA in SAR.

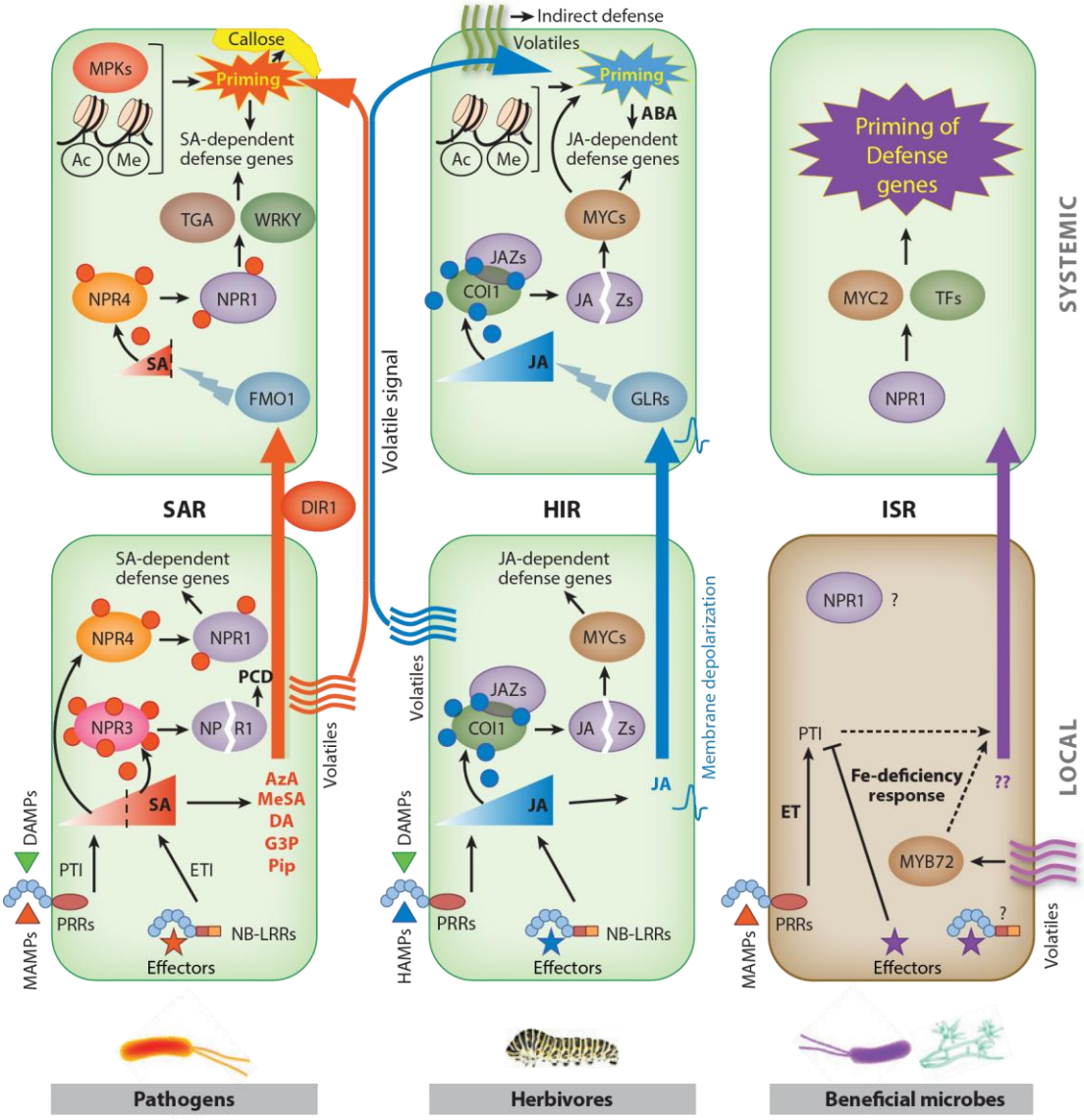

Figure 2-3: Overview of systemic defense responses in plants. Systemic resistance has generally been divided in systemic acquired resistance (SAR) and induced systemic resistance (ISR), which are associated with the presence of pathogenic microbes and root colonization by beneficial microbes respectively. However, insect herbivores can also induce systemic defense and this is called herbivore induced resistance (HIR). SAR: Following pathogen attack, the transcription cofactor NPR1 is needed to initiate defense genes that contribute to SAR. NPR1 occurs as an oligomer in the cytosol and its concentration is regulated by the SA receptors NPR3 and NPR4. After PTI or ETI, SA accumulates and induces a redox change in the cell which facilitates monomerization of NPR1 after which NPR1 is translocated to the nucleus where it interacts with the TGA family of transcription factors, which together with WRKY transcription factors activate SA responsive defense genes. The mobile signal by which SAR is activated in distal tissue is still unknown, MeSA, ROS, dehydroabietinal (DA), lipid derived signaling molecules, glycerol-3-phosphate (G3P)-dependent factor, azelaic acid (AzA), pipecolic acid and peptides have been suggested. The lipid-transfer protein DEFECTIVE IN INDUCED RESISTANCE1 (DIR1) has been shown to be indispensable for G3P, DA and AzA induced activation of SAR and is likely to act as a chaperone for the mobile signal. In distal tissue, SA accumulation has been shown to be critical for SAR establishment. Furthermore, the protein FLAVIN-DEPENDENT MONOOXYGENASE 1 (FMO1) is required in systemic tissue to amplify and transduce the SAR signal from the primary infected leaves. ISR: After root colonization by plant growth promoting rhizobacteria (PGPR) and plant growth promoting fungi (PGPF), plants can become more resistant against pathogens in systemic leaves. Some ISR responses have been shown to be NPR1 dependent. After colonization by ISR inducing plant growth promoting rhizobacteria *MYB72* becomes highly upregulated. *MYB72* is specifically induced in iron limiting conditions. However, *MYB72* is also upregulated after colonization by ISR-inducing PGPR even in non-iron-limiting conditions, which suggests a possible role of Fe ions for the production/translocation of the ISR signal. Besides pathogenic and non-pathogenic microbes inducing SAR and ISR, respectively, insect herbivores can also elicit systemic resistance. This type of systemic resistance is called herbivore induced resistance (HIR).

Defense against insects has generally been associated with JA dependent signaling. JA responses are mostly regulated by the coronatine insensitive 1 (COI1) F-box protein. At high JA concentrations, JA-Ile binds to COI after which JASMONATE-ZIM DOMAIN (JAZ) (a negative regulator of JA dependent signaling) is degraded and the repression of the basic helix-loop-helix Leu zipper transcription factor MYC2 is relieved and defenses downstream activated. Membrane depolarization has been implicated as a mobile signal for HIR. These membrane depolarizations correlated with JA dependent signaling at the systemic tissue. A mutant screening implicates GLUTAMATE RECEPTOR-LIKE (GLR) genes, which encode putative cation channels, in the wound-induced systemic response, suggesting an important role for membrane depolarization in long distance wound signaling. While SAR involves an upregulation of defense genes, ISR does not induce activation of defense mechanisms without the presence of attackers. However, after perception of an attack, defenses are upregulated more strongly. This general response is called priming (Conrath et al., 2006). While ISR is tightly linked with priming, also SAR and HIR responses can be primed by compounds for enhanced SA- and JA dependent responses, respectively. Primed responses may be attributed to epigenetic regulation such as chromatin remodeling or DNA methylation. Hence, plants seem to have the capacity to memorize a stressful situation and subsequently immunize not only themselves but also their offspring. After infection by pathogens or damage by herbivores, plants release volatiles which can induce defenses and defense priming in systemic tissue and neighboring plants. Solid lines depict known interactions, dashed lines are hypothetical interactions. Abbreviations: Ac, acetylation; DAMP, damage-associated molecular pattern; ET, ethylene; ETI, effector-triggered immunity; Fe, iron; HAMP; herbivore-associated molecular pattern; JA, jasmonic acid; MAMP, microbe-associated molecular pattern; Me, methylation; NB-LRR, nucleotide-binding–leucine-rich repeat; PCD, programmed cell death; PRR, pattern-recognition receptor; PTI, PAMP-triggered immunity; SA, salicylic acid; TF, transcription factor.. Adapted from Pieterse et al. (2014).

After root colonization by plant growth promoting rhizobacteria (**PGPR**) and plant growth promoting fungi (**PGPF**), plants can become more resistant against pathogens in systemic leaves. Well known inducers of ISR include *Pseudomonas* spp., *Bacillus* spp., *Trichoderma* spp. and *Piriformospora* spp. Several compounds have been characterized which are known to elicit ISR responses such as lipopolysacharides, flagella, xylanases, cellulases, siderophores, phenazines and proteins and peptides with defense eliciting functions (De Vleesschauwer & Höfte, 2009; Pieterse *et al.*, 2014). Early work on ISR, using the bacteria *Pseudomonas fluorescens* WCS417r, described that ISR, unlike SAR, does not involve the activation of PR proteins and is independent of SA. Additionally, it was shown that rhizobacteria mediated ISR did not involve an increase in the biosynthesis of the plant hormones JA and ET (Pieterse *et al.*, 1996; Pieterse *et al.*, 1998; Pieterse *et al.*, 2000). Further research showed that next to an intact JA and ethylene ET signaling pathway for the induction of ISR, NPR1 is also mandatory for WCS417r-enhanced deposition of callose (Van der Ent *et al.*, 2009). However, De Vleesschauwer & Höfte (2009) reviewed several studies on ISR and the involved defense pathways and found several cases where ISR was SA dependent and/or JA/ET/NPR1 independent, illustrating that ISR may not be solely associated with JA/ET/NPR1 and rather constitutes a broader systemic defense response dependent on the type of inducer. This is also illustrated by the role of NPR1 in ISR. NPR1 functions as the coactivator of SA responsive PR genes, but has also been shown to be required in some instances of ISR, suggesting a dual role for NPR1 (De Vleesschauwer & Höfte, 2009; Pieterse *et al.*, 2014).

Knockout studies revealed *MYB72* to be crucial for the onset of ISR (Segarra *et al.*, 2009). The MYB transcription factor gene *MYB72* is little expressed in control plants, but after

colonization by ISR inducing plant growth promoting rhizobacteria becomes highly upregulated. Interestingly, *MYB72* is specifically induced in iron limiting conditions. However, *MYB72* is also upregulated after colonization by ISR-inducing PGPR, even in non-iron-limiting conditions, which prompted Pieterse *et al.* (2014) to suggest a possible role of Fe ions for the production/translocation of the ISR signal.

Under iron limiting conditions, rhizobacteria such as *P. fluorescens* WCS417r and, *P. aeruginosa* 7NSK2 have been shown to produce SA-containing siderophores. These bind with Fe^{3+}, forming soluble complexes, which can be sequestered in the microbial cell, thereby competing for Fe ions with deleterious rhizobacteria (Höfte & Bakker, 2007). In vitro studies showed that SA concentration also increased under iron limiting conditions, so it has been argued that the bacterial SA production elicits defense responses in plants (De Meyer & Höfte, 1997; De Meyer *et al.*, 1999a; De Meyer *et al.*, 1999b). While SA production by bacteria has been confirmed *in vitro*, *in situ* measurements have been more difficult (Bakker *et al.*, 2014). It has been shown by Audenaert *et al.* (2002b) in tomato, using mutant lines that rather a synergistic interaction between the siderophore pyochelin and the phenazine compound, pyocyanin, contributed to *P. aeruginosa* 7NSK2 mediated ISR, rather than SA alone. The same study additionally showed that ISR by these rhizobacteria could not be induced in the *NahG* tomato mutants, which are unable to accumulate free SA, illustrating that ISR is not solely dependent on JA/ET in plants.

Further downstream, gene expression analysis of *Arabidopsis* revealed that *MYC2* was consistently upregulated in ISR-expressing plants and that knockout mutants were unable to mount WCS417r-ISR against the pathogens Pst DC3000 and *Hyaloperonospora parasitica*, suggesting that MYC2 is an important regulator in rhizobacteria-mediated ISR (Pozo *et al.*, 2008).

Besides pathogenic and non-pathogenic microbes inducing SAR and ISR, respectively, insect herbivores can also elicit systemic resistance. This type of systemic resistance is called herbivore induced resistance (**HIR**) (Figure 2-3). Defense against insects has generally been associated with JA dependent signaling (Nguyen *et al.*, 2016). JA responses are mostly regulated by the coronatine insensitive 1 (**COI1**) F-box protein. At high JA concentrations, JA-Ile binds to COI after which JASMONATE-ZIM DOMAIN (**JAZ**) (a negative regulator of JA dependent signaling) is degraded and the repression of the basic helix-loop-helix Leu zipper transcription factor MYC2 is relieved and defenses downstream activated (Robert-Seilaniantz *et al.*, 2011). Interestingly, while JA has generally been thought of to be the mobile signal in HIR, in systemic tissue also SA responsive PR1 gene was upregulated. Furthermore, experiments with different mutants revealed that HIR was not exclusively regulated by SA, JA and ET suggesting a separate pathway or pointing to an interplay of several signals (De Vos *et al.*, 2006; Vos *et al.*, 2013). Additionally, membrane

depolarization has been implicated as a mobile signal for HIR. After the wounding of *Arabidopsis* leaves, wound-activated surface potential changes were observed which propagate to systemic tissue. These membrane depolarizations correlated with JA dependent signaling at the systemic tissue. A mutant screening showed that mutations in GLUTAMATE RECEPTOR-LIKE (**GLR**) genes, which encode putative cation channels, have an attenuated wound-induced systemic response, suggesting an important role for membrane depolarization in long distance wound signaling (Mousavi *et al.*, 2013).

While SAR involves an upregulation of defense genes, ISR does not induce activation of defense mechanisms without the presence of attackers. However, after perception of an attack, defenses are upregulated more strongly. This general response is called priming (Conrath *et al.*, 2006). While ISR is tightly linked with priming, also SAR and HIR responses can be primed by compounds for enhanced SA- and JA dependent responses, respectively. Thus, priming encompasses both SAR, HIR and ISR and constitutes a state in which defense are prepared to respond more strongly at the advent of a future attack (Conrath *et al.*, 2015). In section 2.3.3, we will further investigate different priming mechanisms.

Most research on SAR/ISR focuses on signaling confined to vascular signaling. However, it should be noted that plants release biogenic volatile organic compounds (BVOCs) in response to an attack by pathogens, insects or beneficial microbes (Engelberth *et al.*, 2004; Yi *et al.*, 2009; Pineda *et al.*, 2013). Some of these BVOCs can also induce a priming response and PR expression in systemic tissue and even in neighboring plants (see Chapter 3 and Chapter 4) which may indicate that BVOCs also act as a mobile signal for systemic resistance offering several benefits compared to vascular signals (Heil & Ton, 2008).

Because of the overlap and fuzzy boundaries between ISR, SAR and HIR, it raises the question whether the distinction between these types of systemic resistance is maintainable. Analogous to the zigzag model, perhaps we should rethink the concept of SAR, ISR and HIR in the light of new insights and speak of systemic resistance and priming in general, or make the distinction based of the type of attacker i.e. herbivore induced resistance (**HIR**), pathogen induced resistance (**PIR**), beneficial microbe induced resistance (**BIR**) and volatile induced resistance (**VIR**).

2.3.2 Priming

As mentioned above, priming constitutes a state in which defenses are prepared to respond more strongly at the advent of a future attack. Priming offers a mechanism which combines increased disease resistance and low allocation costs. As stated by Martinez-Medina *et al.* (2016): “Defense priming is postulated to be an adaptive, low-cost defensive measure because defense responses are not, or only slightly and transiently activated by a given priming stimulus. Instead, defense responses are deployed in a faster, stronger, and/ or

more sustained manner following the perception of a later challenging signal (the triggering stimulus) in times of stress" (Figure 2-4). Thus, the great advantage of priming is a stronger or faster activation while limiting the cost and damage endured during the time needed to mount a defense compared to nonprimed plants (Frost *et al.*, 2008).

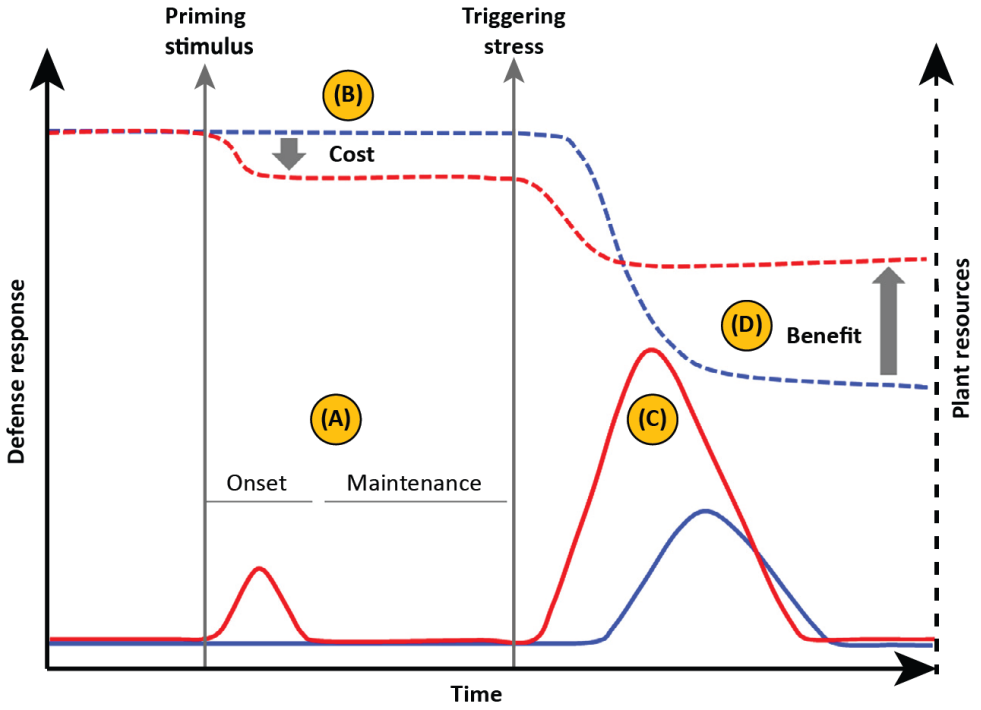

Figure 2-4: Diagram depicting the difference between a primed and nonprimed defense response. Solid lines depict the defense response of the plant, while the dashed lines depict the pool of plant resources. Red lines represent primed plants, while blue lines represent nonprimed plants. After a priming stimulus, defense responses are only transiently and/or lightly induced (A), this corresponds to a low resource cost in primed plants (B). At the onset of a stress trigger (e.g. fungal or bacterial pathogen, insect herbivory) primed plants will exhibit a stronger defense response compared to nonprimed plants (C). This coincides with a greater resource cost in nonprimed plants compared to primed plants (D). Adapted from Martinez-Medina *et al.* (2016)

The lower resource costs were illustrated by van Hulten *et al.* (2006), they showed that the induction of defense in *Arabidopsis* by β-aminobutyric acid (BABA) or BTH had a negative impact on both the relative growth rate and the seed production, whereas constitutively primed in enhanced disease resistance mutant plants (*edr1-1)* did only exhibit a minor effect on the relative growth rate and had no effect on the seed production. Furthermore, *edr1-1* mutants showed an equal level of protection compared to BTH-treated wild-type plants and constitutively activated defense mutants. Thus, while still offering an equal level of protection, allocation costs are less in primed plants, compared to plants where defense is constitutively induced, illustrating the benefits of defense priming.

Primed plant responses are manifold and include effects such as increased callose deposition (Ton & Mauch-Mani, 2004; Hamiduzzaman *et al.*, 2005), increased extrafloral nectar production which serves as an attractant for natural enemies of plant herbivores (Kost & Heil, 2006), increased BVOC production (Engelberth *et al.*, 2004; Frost *et al.*, 2008b),

increased plant defense hormone production (Engelberth *et al.*, 2004; Frost *et al.*, 2008b; Jung *et al.*, 2009; Gamir *et al.*, 2012; Scalschi *et al.*, 2013) and increased production of PR proteins (Yi *et al.*, 2009; Kravchuk *et al.*, 2011).

Several compounds have been shown to act as priming agents for enhanced defense (Table 2-1). Some of these compounds which are known to activate plant defense signaling, can (at low concentrations) also prime plant defense for a higher induction of plant defense after a subsequent biotic attack. An interesting group includes the plant volatiles released after insect herbivore- or pathogen attack which prime neighboring plants to prepare for an impending attack. Pioneering work was done by Engelberth *et al.* (2004); upon exposure of maize seedlings to herbivore induced plant volatiles (**HIPVs**), they observed a higher production of the plant defense hormone JA. The priming effect of HIPVs was also found to enhance defense against insect herbivores (Ton *et al.*, 2007; Frost *et al.*, 2008a). Within the HIPVs, GLVs constitute a large group and have been shown to induce defenses against insect herbivores. In Chapter 3, we will elaborate on the role of GLVs in plants and the interaction with their environment.

481 **Table 2-1: Non exhaustive overview of compounds which elicit a priming response on different plant species.** Priming by prior exposure to avirulent pathogens, or
482 beneficial microbes is not included.

Priming agent	Concentration (mM)	Application	Plant species	Challenge	Reference
Indole	5.12 x 10^{-6}	Aerial	*Zea mays*	*Spodoptera littoralis*	(Erb *et al.*, 2015)
GLVs	1.2 x 10^{-6}	Aerial	*Populus deltoides X nigra*	*Lymantria dispar*	(Frost *et al.*, 2008b)
	5 x10^{-6}	Aerial	*Zea mays*	*Spodoptera exigua*	(Engelberth *et al.*, 2004; Engelberth *et al.*, 2007)
	84.73	Foliar spray	*Camelia sinensis*	*Ectropis obliqua*	(Xin *et al.*, 2015)
Pipecolic acid	1	Soil drench	*Arabidopsis thaliana*	*Pseudomonas syringae*	(Navarova *et al.*, 2012)
BTH induced volatiles	*ND*		*Phaseolus lunatus*	*Pseudomonas syringae*	(Yi *et al.*, 2009)
Hexanoic acid	1	Soil drench	*Arabidopsis thaliana*	*Botrytis cinerea*	(Kravchuk *et al.*, 2011)
	0.6	Soil drench	*Solanum lycopersicum*	*Botrytis cinerea,* *Pseudomonas syringae*	(Vicedo *et al.*, 2009; Scalschi *et al.*, 2013)
	1	Soil drench	*Citrus clementina*	*Alternaria alternata*	(Llorens *et al.*, 2016)
Azelaic acid	1	Foliar spray	*Arabidopsis thaliana*	*Pseudomonas syringae*	(Jung *et al.*, 2009)

β-aminobutyric acid	*0.15-0.3*	*Soil drench*	*Arabidopsis thaliana*	*Hyaloperonospora arabidopsis, Alternaria brassisicola, Plectophaerella cucumerina, Peronospora parasitica*	(Zimmerli *et al.*, 2000; Ton & Mauch-Mani, 2004; Van der Ent *et al.*, 2009)
	0.5 mM	Floating leaf disk assay	*Vitis vinifera*	*Plasmopara viticola*	(Hamiduzzaman *et al.*, 2005)
	10	Foliar spray	*Solanum tuberosum*	*Phytophthora infestans*	(Floryszak-Wieczorek *et al.*, 2015)
HIPV	ND	Aerial	*Zea mays*	*S. littoralis*	(Ton *et al.*, 2007)
	ND	Aerial	*Phaeseolus lunatus*	Wounding, *Tetranychus urticae*	(Choh & Takabayashi, 2006; Kost & Heil, 2006)
BTH	0.1	Foliar spray	*Arabidopsis thaliana*	*Pseudomonas syringae*	(Kohler *et al.*, 2002)
Silicon	*2*	Hydroponic	*Oryza sativa*	*Cochliobolus miyabeanus Cnaphalocrocis medinalis*	(Ye *et al.*, 2013; Van Bockhaven *et al.*, 2015a)

Abbreviations: GLV: green leaf volatile, HIPV: herbivore induced plant volatiles; BTH: benzo-(1,2,3)-thiadiazole-7-carbothiolic acid S-methyl ester; MeJA: methyl jasmonate; ND: not determined

Most research has focused on priming compounds which are translocated through the vascular system and induce resistance in distal plant tissue. However, volatile signals offer several benefits over vascular signals in plant defense: it is faster, as BVOCs are released within minutes and is not limited by physical (vascular) constraints (Li & Blande, 2017). Heil & Ton (2008) proposed a two-step regulation model for systemic resistance by an interplay of airborne and vascular signaling (Figure 2-5). After a leaf is attacked by a pathogen or insect, BVOCs can form within minutes (Fall *et al.*, 1999). These BVOCs can reach neighboring leaves and even neighboring plants. At high concentrations, plant defense can be induced. However, because of aerial diffusion and eddy currents, concentrations of BVOC at the receiver leaf may have dropped to priming concentrations. After leaves are primed, defense will be enhanced after the vascular signal reaches the targeted tissue after 6 to 24h (Shah & Zeier, 2014). Thus, systemic resistance may be acquired through an interplay between volatile and vascular signals.

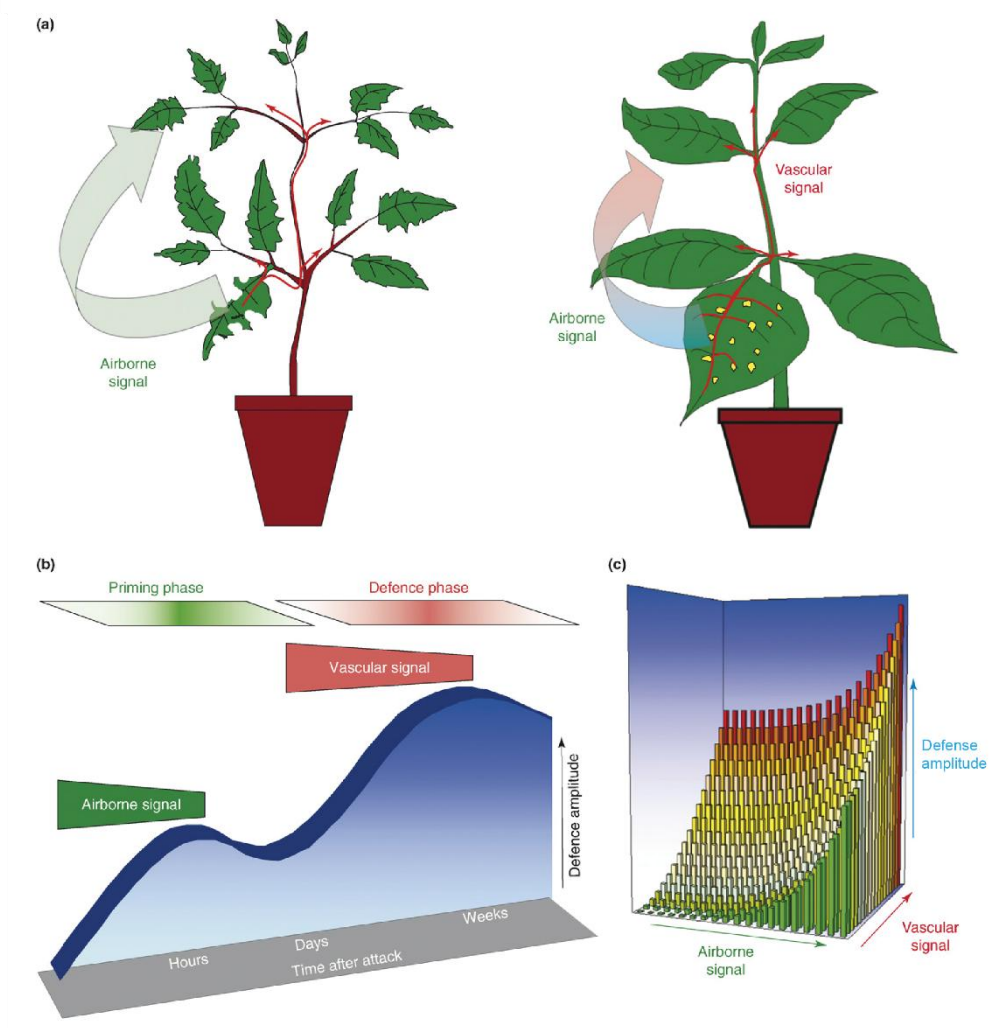

Figure 2-5: Two step regulation model for systemic resistance by volatile and vascular signaling. (a) After an attack by a caterpillar (left) or pathogen (right), volatile signals are released within minutes or hours, and can reach and prime distal plant parts coinciding with (b) a small induction of defense. These signals are followed by the vascular signal (days-weeks) that can boost the defense expression, resulting in enhanced defense. Heil & Ton (2008)

2.3.3 Molecular mechanisms of priming

While the exact mechanisms of plant priming have not yet been elucidated, we are slowly beginning to uncover several possible mechanisms. Evidence points to a role for mitogen-activated protein kinase (**MPK**) pathways, storage of compounds, and epigenetic mechanisms. Here, we will give a concise overview of different mechanisms which have been attributed to the priming response.

A first hypothesis explaining the priming response is the accumulation of dormant enzymes involved in signal transduction and amplification (Figure 2-6). Because of the central role in signal amplification, the MPK signaling cascades are possible candidates for the priming response. After perception of a MAMP or DAMP, MPK kinase kinases (**MPKKKs**) are activated after which they activate MPKKs by phosphorylation which in turn activate MPKs and ultimately activate genes involved in plant defense (Conrath, 2011). The involvement of MPKs in the priming response in *Arabidopsis* has been shown by Beckers *et al.* (2009). They found that after priming by BTH, MPK3 and MPK6 mRNA accumulated. This coincided with an increased presence of inactive MPK3 and MPK6. After challenge with virulent *Pseudomonas syringae* pv. *malulicola*, primed plants exhibited a stronger activation of MPK3 and 6. Furthermore, the priming response was lost in mpk3 and mpk6 mutants, illustrating a critical role of these MPKs in the priming process. While the involvement of MPK cascades can explain primed response at a short timescale (hour-days), it has been debated whether this is sufficient to justify priming on longer timescales (Pastor *et al.*, 2013).

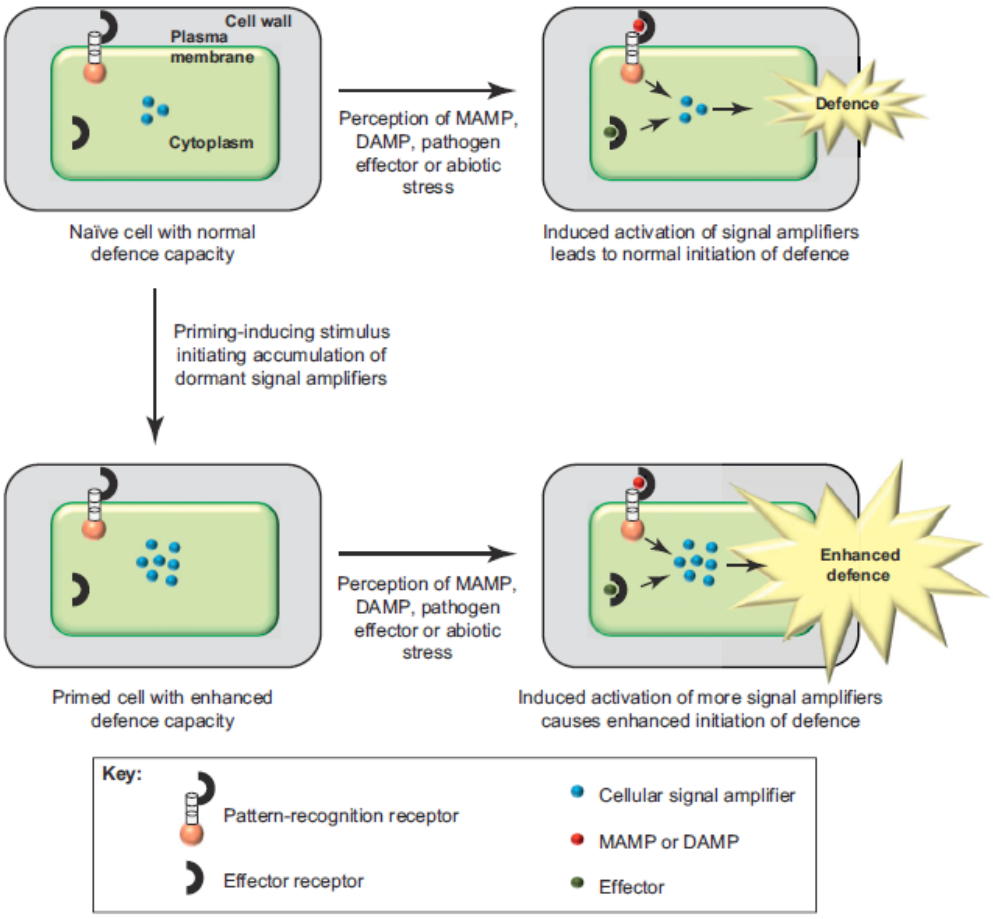

Figure 2-6: Diagram illustrating the involvement of inactive cellular signal amplifiers in the primed response of plants. A priming inducing stimulus can enhance the cellular level of inactive cellular signal proteins. After subsequent exposure to a DAMP or MAMP, more of these inactive signal amplifiers are activated compared to nonprimed cells. Abbreviations: MAMP: microbe-associated molecular pattern; DAMP: damage associated molecular pattern. (Conrath, 2011).

Epigenetic mechanisms such as histone modifications and DNA methylation may provide a mechanistic basis for priming on a longer timescale and transgenerational priming (Conrath *et al.*, 2015; Espinas *et al.*, 2016). In the nucleosome, 147 base pairs of genomic DNA are tightly wrapped around an octamer of histone proteins. In this state, the genes are genetically inactive. During the priming process, covalent modification of the histones can occur which loosens the ionic interactions between the histones and the DNA, and additionally providing a docking site for transcription co-activators or other proteins such as RNA polymerase II, which will lead to enhanced gene expression (Figure 2-7) (Conrath, 2011). Jaskiewicz *et al.* (2011) demonstrated that priming by BTH in *Arabidopsis* could be attributed to histone modification of the promoter of WRKY29. The BTH treatment or the histone modifications did not induce transcription of WRKY29. However, upon challenge with water infiltration, a higher upregulation of WRKY29 was found.

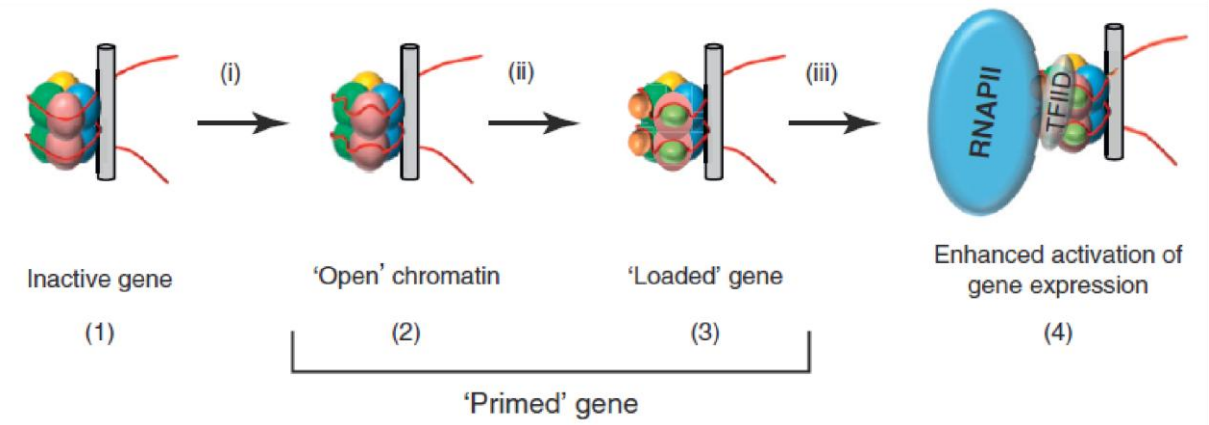

Figure 2-7: Diagram depicting the possible role of chromatin modifications in the priming process. (1) When DNA is tightly wrapped around histones, genes are transcriptionally inactive. (2) A priming stimulus can induce covalent modifications of the histones to reduce its ionic interaction with the DNA and (3) provide easier docking of transcription co-activators or other effector proteins on chromatin. Binding of these effector proteins loads the appropriate gene, (4) enhancing later gene expression (Conrath, 2011).

Besides histone modification, DNA methylation constitutes another epigenetic mechanism to affect gene transcription. This involves methylation of cytosine in the DNA which generally leads to a downregulation of the activation of defense genes (Espinas *et al.*, 2016; Zhu *et al.*, 2016). However, DNA methylation can also result in enhanced defense as was shown for rice in the defense against the pathogenic fungus *Magnaporthe oryzae*. Li *et al.* (2011) showed that the promoter region of the *Pib* gene, which plays a role in disease resistance, was heavily cytosine methylated which did not disappear after induction of *Pib*. In addition, demethylation of the promoter region led to increased susceptibility to *Magnaporthe grisea*, suggesting a role for DNA methylation in the disease resistance.

While both DNA methylation and histone modifications have long lasting effects on gene transcription within one generation, only DNA methylation is transferable through meiosis and is therefore a more plausible mechanism for transgenerational priming (Pastor *et al.*, 2013; Conrath *et al.*, 2015). Ali *et al.* (2013) also found evidence for a role of methylation in the primed response on the expression of Bowman-Birk type trypsin inhibitor (TI). In their study they exposed maize plants to volatiles released by conspecific plants which were infested with *Mythimna separata*. When *M. separata* larvae fed on these pre-exposed plants, they exhibited reduced larval development. This response could be attributed to enhanced expression of TI. Further investigation revealed that previous exposure to the plant volatiles resulted in demethylation of the promotor region of TI, possibly allowing for a faster upregulation. Another proposed mechanism for defense priming is the accumulation of inactive metabolite conjugates, which become activated upon challenge by a stressor. These can comprise pyto-anticipins such as glucosinolates and benzoxazinoids which are activated upon hydrolysation by glucosidases in the cytoplasm. Furthermore, plant hormones can also

occur in glucosylated forms and stored in the vacuole such as glucosylated abscicic acid and salicylic acid 2-O-beta-D-glucose (Pastor *et al.*, 2013; Pastor *et al.*, 2014).

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Chapter 3 Green Leaf Volatile Production by Plants: a Meta-Analysis

Adapted from: Ameye M, Allmann S, Verwaeren, J, Haesaert G, Smagghe G, Schuurink R, Audenaert K (2017) Green leaf volatile production by plants: a meta-analysis. New Phytologist, doi:10.1111/nph.14671

3.1 Abstract

Plants respond to stress by releasing biogenic volatile organic compounds (BVOCs). Green Leaf Volatiles (GLVs), which are abundantly produced across the plant kingdom, comprise an important group within the BVOCs. They can repel or attract herbivores and their natural enemies; they can induce plant defenses or prime plants for enhanced defense against herbivores and pathogens and can have direct toxic effects on bacteria and fungi.

Unlike other volatiles, GLVs are almost instantly released upon mechanical damage and (a)biotic stress and could thus function as an immediate and informative signal for many organisms in the plant's environment. We used a **meta-analysis** approach in which literature data on GLV production during biotic stress responses were compiled and interpreted. We identified that different types of attackers and feeding styles add a level of complexity to the amount of emitted GLVs, compared to wounding alone. This meta-analysis illustrates that there is less variation in the GLV profile than we initially presumed, that pathogens induce more GLVs than insects and wounding and that there are clear differences in GLV emission between monocots and dicots.

Besides the meta-analysis, this review provides an update on recent insights into the perception and signaling of GLVs in plants.

3.2 Introduction

Green leaf volatiles represent an important group of plant volatiles. They consist of six carbon (C_6) compounds including alcohols, aldehydes and esters (Dudareva *et al.*, 2006) and are released from almost every plant. Curtius & Franzen (1914) were the first to isolate *E*-2-hexenal (E-2-HAL) from 600 kg of European hornbeam leaves. The release of GLVs is caused by mechanical damage (Halitschke *et al.*, 2004b) or herbivory (Allmann *et al.*, 2013), by fungal or bacterial infection (Piesik *et al.*, 2011b; Ponzio *et al.*, 2013), but also as a consequence of abiotic stress such as drought (Wenda-Piesik, 2011), heat (Copolovici *et al.*, 2012), high light (Loreto *et al.*, 2006) and presence of heavy metals (Obara *et al.*, 2002) (Figure 3-1). This general response after (a)biotic stress suggests an important role for GLVs in plants in reaction to stressful environments. GLVs are implicated in a panoply of interactions; they have been reported to repel or attract herbivores and their natural enemies (Visser *et al.*, 1979; Turlings *et al.*, 1991; Scala *et al.*, 2013a), to activate and prime plant defenses (Engelberth *et al.*, 2004; Ameye *et al.*, 2015)(see Chapter 4), to activate abiotic-stress related genes (Yamauchi *et al.*, 2015), and to have antibacterial and antifungal properties (Nakamura & Hatanaka, 2002; Kishimoto *et al.*, 2008) (Figure 3-1).

The widespread GLV production as a general response upon different types of biotic stress prompts the question, whether the type of biotic attack moulds the outcome and pattern of GLV production.

To answer this question, we performed a meta-analysis on available literature. In order to get a view on the effect of a treatment on the physiology of the plant; traditionally, reviews are written. However, such an approach often lacks a holistic, generalized, systematic dimension. A meta-analysis offers the benefit of providing a quantitative synthesis on available data or literature and allows to assess between-study variability and identify factors which determine the treatment effects on plant physiology (Trikalinos *et al.*, 2008).

The analysis reveals a clear interaction between GLV production, the type of biotic attacker, the type of feeding guild and the class of plants. In addition to the meta-analysis, we give an update on the current status of GLV research regarding biosynthesis, perception and signal transduction, and discuss the biological function of GLVs.

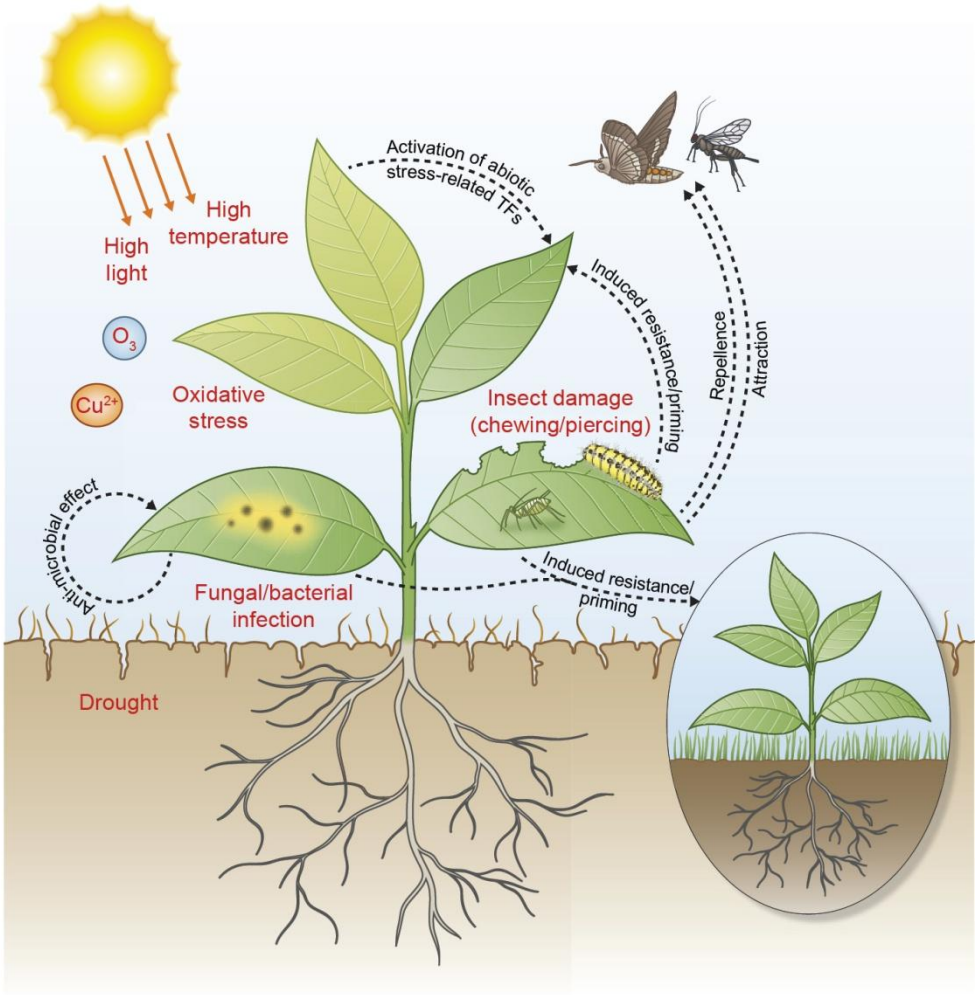

Figure 3-1: Overview of the different functions of GLV. Overview of the different types of (a)biotic stress which drive GLV production (red) and reported functions of GLVs (black). See text for references.

3.3 Biosynthesis

GLVs are generated through the oxylipin-pathway from C18-polyunsaturated fatty acids (FAs; α-linolenic acid (ALA) and linoleic acid; Figure 3-2) (Matsui, 2006). While it has been assumed that FAs are hydrolysed from membrane lipids (Matsui *et al.*, 2000) prior to further enzymatic reaction, only little is known about the identity of specific lipases that play a role in the liberation of FAs for the GLV pathway (Mwenda & Matsui, 2014). In *Arabidopsis*, the supply of free FAs for the biosynthesis of the plant hormone JA, which is formed via another branch of the oxylipin-pathway, is regulated by a number of different lipases, all belonging to the class of phospholipases-A1 (Bonaventure, 2014). These lipases can act in different tissues (Ellinger *et al.*, 2010), upon different stimuli (Ellinger & Kubigsteltig, 2010) and at different stages of the stress-response. One of them, phospholipase A-Iγ1, is involved in the early wound-induced accumulation of JA and OPDA (Ellinger *et al.*, 2010). Whether this lipase can also supply substrate for the HPL branch of the oxylipin pathway has not been

tested yet. In *Nicotiana attenuata* glycerolipase A1 (GLA1) is the major supplier of free FAs for the formation of JA after herbivory, but is not involved in the biosynthesis of GLVs (Bonaventure *et al.*, 2011) So far, only two lipases have been implicated in the formation of GLVs, phospholipase Dα4 and Dα5 (**OsPLDα4/5**); antisense expression of OsPLDα4/5 in rice reduced not only the levels of α-linolenic acid and JA but also the release of the GLVs *Z*-3-hexenal (**Z-3-HAL**) and *Z*-3-hexenol (**Z-3-HOL**) after herbivory or wounding (Qi *et al.*, 2011). However, this study did not unambiguously show whether PLDα4/5 can directly supply free FAs for the GLV pathway or whether they only indirectly affect GLV levels by hampering JA-biosynthesis which can feed-back on GLV biosynthesis (Wei *et al.*, 2011). From recent research in *Arabidopsis* we know that, in damaged tissue, GLVs can also be formed without cleavage from membrane lipids (Nakashima *et al.*, 2013) and thus via a lipase-independent pathway. Free or membrane bound FAs are dioxygenated by 13-lipoxygenases (**LOX**), which are non-heme, iron-containing enzymes catalyzing the incorporation of molecular oxygen at the C-13 position (Liavonchanka & Feussner, 2006; ul Hassan *et al.*, 2015) of polyunsaturated fatty acids. The resulting 13-hydroperoxy fatty acids (**13-HPs**) are a common substrate for different enzymes including the two cytochrome P450s allene oxide synthase (**AOS**) and hydroperoxide lyase (**HPL**; Gobel & Feussner, 2009; Savchenko *et al.*, 2014). Both AOS and HPL are members of the CYP74 family, an atypical subgroup within the P450 family. The CYP74 family is involved in the metabolism of hydroperoxides, and does not require oxygen, nor an NADPH-reductase, resulting in high turnover numbers (Noordermeer *et al.*, 2001; Hughes *et al.*, 2009). Furthermore, evidence shows that CYP74 genes are highly conserved and were present in the last common ancestor of plants and animals, but that these were lost in all metazoan lineages with the exception of Placozoa, Cnidaria and Cephalochordata (Lee *et al.*, 2008).

Because of the shared substrate, plants need to distinctly regulate GLV and JA biosynthesis. As stated by Mochizuki *et al.* (2016) there are two ways to accomplish this. Firstly, by a distinct spatiotemporal expression of *HPL* and *AOS* (Mwenda et al., 2015), and secondly, by expression of pathway-specific LOXs. While some plant species possess pathway-specific LOXs that feed 13-HPs either only into the AOS-branch, leading to the formation of JA, or into the HPL-branch, leading to the formation of GLVs (Allmann *et al.*, 2010; Christensen *et al.*, 2013b), other plant species have LOXs that can supply substrate to both pathways (Wang *et al.*, 2008b; Mochizuki *et al.*, 2016).

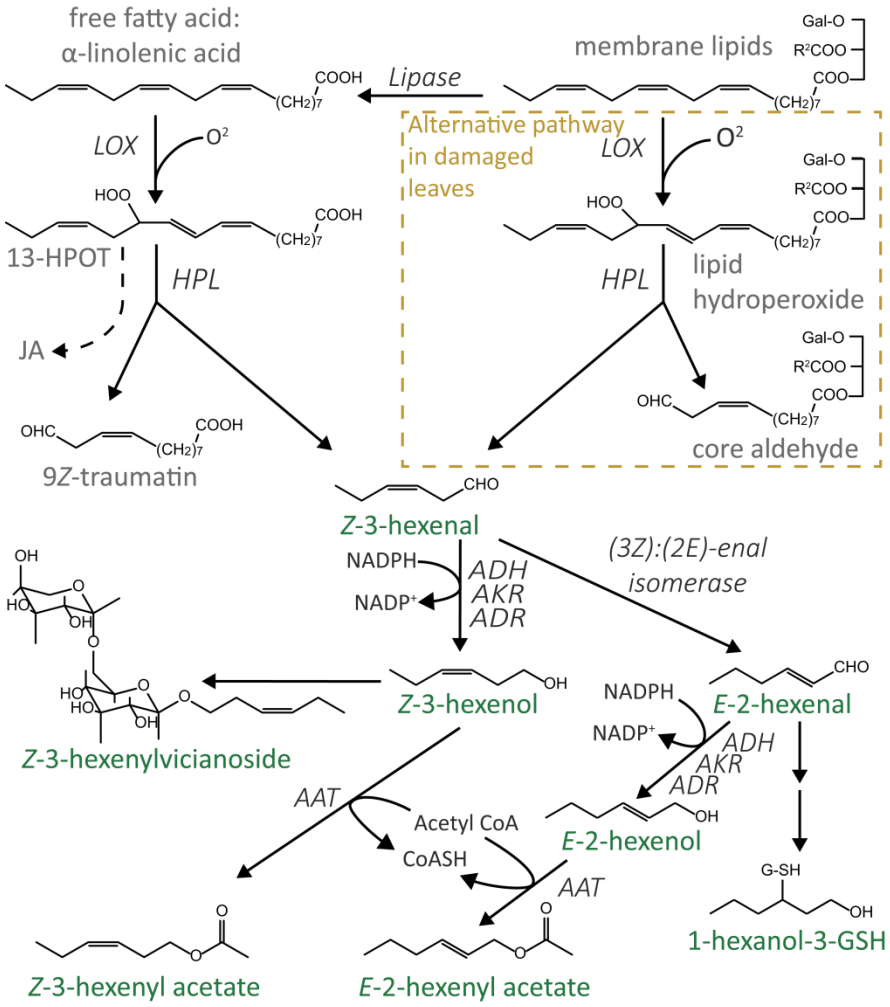

Figure 3-2: Biosynthetic pathway of green leaf volatiles. Free fatty acids (FAs) are released from membrane lipids. The two FAs that serve as substrate for the formation of saturated and unsaturated C_6-volatiles are linoleic acid and α-linolenic acid, respectively. This figure focuses on the formation of the most abundant unsaturated C_6-volatiles. For more detailed information on the biosynthesis of GLVs see recent reviews (Mwenda & Matsui, 2014; Hassan *et al.*, 2015). Molecular oxygen is introduced into α-linolenic acid and the resulting hydroperoxide (13-HPOT) is cleaved into a C12- ((9*Z*)-traumatin) and a C6- ((*Z*)-3-hexenal) compound. 13-HPOT is substrate for the different branches of the oxylipin pathway and is thus also used for the formation of jasmonic acid. In damaged leaf tissue galactolipids can directly be oxygenated without prior cleavage of the free FAs. The resulting lipid hydroperoxide is cleaved into a traumatin containing galactolipid and (*Z*)-3-hexenal. (*Z*)-3-hexenal is quickly reduced to (*Z*)-3-hexenol and can be glycosylated or converted to (*Z*)-3-hexenyl acetate. Some plants and insects possess a (3*Z*):(2*E*)-enal isomerase that catalyzes the formation of (*E*)-2-hexenal from (*Z*)-3-hexenal. (*E*)-2-hexenal can be further converted to its alcohol and acetate, or it can be conjugated to glutathione (GSH). LOX, lipoxygenase; HPL, hydroperoxide lyase; ADH, alcohol dehydrogenase; AKR, aldo-keto reductase; ADR, aldehyde reductase; AAT, alcohol acetyl transferase Adapted from Nakashima *et al.* (2013).

Competitive substrate flux into the AOS- and HPL-branch has already been reported for several plant species by silencing or overexpression of the HPL/AOS pathways (Halitschke *et al.*, 2004a; Liu *et al.*, 2012; Tong *et al.*, 2012; Xin *et al.*, 2014).

When fed into the HPL-branch, 13-HPs are cleaved into 12 carbon oxoacids, like (9*Z*)-traumatin, and a C6 compound. Depending on whether α-linolenic acid or linoleic acid is the substrate for HPL, the resulting C6 compound is either the unsaturated Z-3-HAL or the saturated n-hexanal, respectively. Z-3-HAL is relatively unstable and thus re-arranges either

spontaneously or enzymatically to (*E*)-2-hexenal (E-2-HAL; Noordermeer *et al.*, 1999; Allmann & Baldwin, 2010; Kunishima *et al.*, 2016). In previous literature the enzyme responsible for this re-arrangement has been defined as (3*Z*):(2*E*)-enal isomerase. Although, there is some disagreement within the scientific community on this terminology we will use, for the sake of brevity and uniformity, the term (3*Z*):(2*E*)-enal isomerase and the abbreviation HI (hexenal isomerase) for this double bond migration. While extensive research has been done on plant LOXs and HPLs (Andreou & Feussner, 2009; Hughes *et al.*, 2009; Mwenda & Matsui, 2014; ul Hassan *et al.*, 2015), much less is known about the enzymes that are involved in the reduction of C6-aldehydes to their corresponding alcohols. For many years it was assumed that this conversion was catalyzed by an alcohol dehydrogenase (**ADH**; Hatanaka, 1993), however, an *Arabidopsis* mutant lacking ADH activity could not fully abolish the levels of Z-3-HOL, indicating that there is either gene redundancy or that there are other enzymes involved as well. More recent research revealed that NADPH-dependent aldehyde- or aldo-keto reductases play a role in this conversion (Matsui *et al.*, 2012). Subsequently, alcohols can be modified by the activity of an alcohol acyltransferase (AAT; D'Auria et al., 2007) to their corresponding esters.

3.4 Meta-analysis

Since the production of GLVs often has been reported in studies investigating the headspace of plants after wounding or herbivore attack (Shiojiri *et al.*, 2006a), GLVs have mainly been associated with infestations by herbivorous insects (from now on referred to as herbivores). However, in the last decade evidence has accumulated that also pathogens can induce the release of GLVs (Piesik *et al.*, 2011b; Scala *et al.*, 2013a). To obtain a better insight into the production pattern of GLVs, we performed a meta-analysis on 51 studies (163 treatments), investigating GLV production of plants after herbivory, fungal infection or mechanical damage. Following the procedure described in Rowen & Kaplan (2016), we calculated the treatment effect (TE) of each study, using Hedges' g, which represents the standardized difference of the means between the treatment and the control (Figure Box 2)(Hedges, 1981). Details of the meta-analysis can be found in section 3.9. Studies regarding the effect of abiotic stress or bacterial, viral or oomycete infection on GLV production were not included in this meta-analysis as they did not meet all the requirements.

Hedges' g (Hedges, 1981), represents the standardized mean difference between two populations, or in our case, between two treatments. The equation is as follows:

$$g = \frac{\overline{x_1} - \overline{x_2}}{s^*}$$

$$s^* = \sqrt{\frac{(n_1 - 1){s_1}^2 + (n_2 - 1){s_2}^2}{n_1 + n_2 - 2}}$$

Where, $\overline{x_1}$ and $\overline{x_2}$ represent the mean of the treatment and the control, respectively. s^* represents the pooled standard deviation of the treated and control means. n_1 and n_2 represent the number of replicates in the treated and control treatments, respectively and s_1 and s_2, represent the standard deviation of the treated and control treatments, respectively. The main difference with comparing means of two populations is that Hedges' g is unitless, which allows the direct comparison of data between different studies.

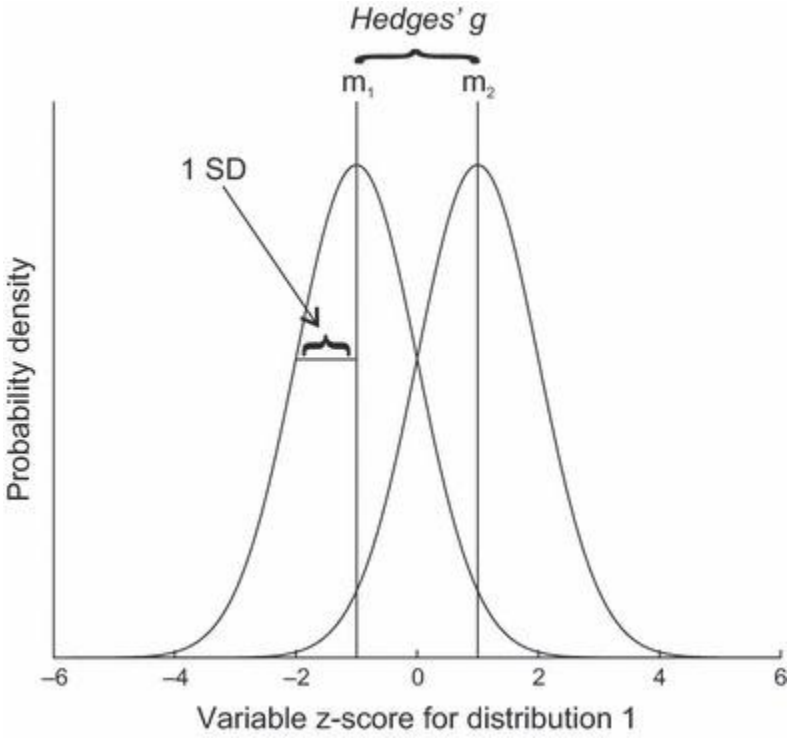

Figure Box 2: Hedges' g. Illustration of the effect size of two hypothetical populations (Hentschke & Stüttgen, 2011)

3.4.1 The type of stress influences the total amount of GLVs released

Our meta-analysis showed that the amount of GLVs produced differs depending on the type of stress the plant encounters. While plants infected by fungi showed a greater treatment effect (TE = 9.67, P < 0.001) than wounded plants (TE = 4.69, P < 0.001), the effect after herbivory was smaller (TE = 1.86, P < 0.001) (Figure 3-3a, Table 3-3, Table 3-5, Table 3-6, Table 3-7)

The prompt formation after wounding and the fact that GLVs are formed from plant endogenous components advocate in favor of a role of GLVs as damage associated molecular patterns (**DAMPs**) (Duran-Flores & Heil, 2016). However, while both fungal pathogens and herbivores also inflict cellular damage, treatment effects are significantly different from the wounding treatments. This disparity points to an important role for fungi or herbivores in modulating the GLV production. As suggested by Duran-Flores & Heil (2016) plants can make use of a complex mix of “danger signals” to recognize the nature of the attacker. This mix might consist of endogenous DAMPs and enemy derived signals, including herbivore associated molecular patterns (**HAMPs**) or MAMPs. The following paragraphs will discuss the possible role of HAMPs and MAMPs in modulating the wound-induced GLV release

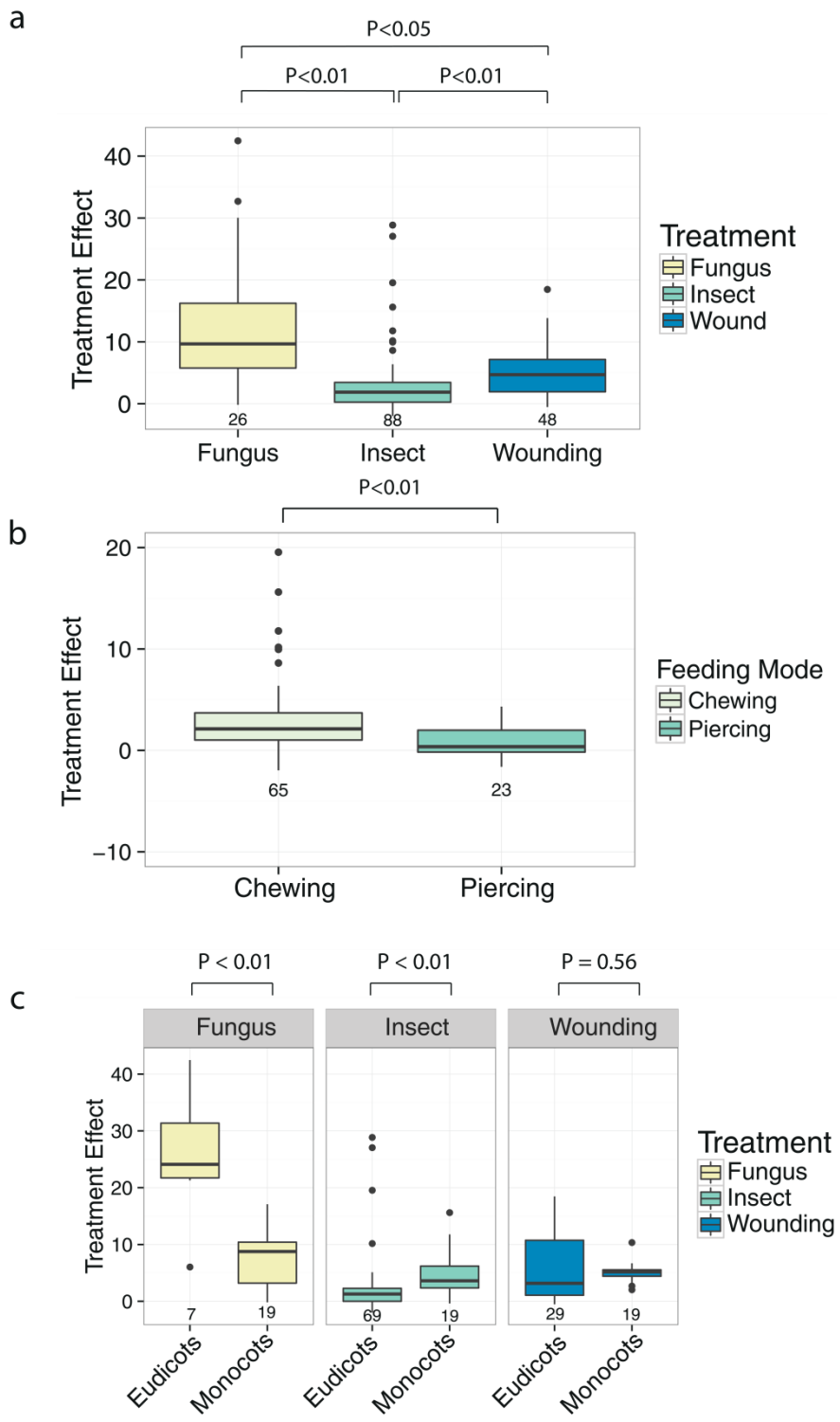

Figure 3-3: Effect size on the total production of GLVs in response to different treatments. a: The type of treatment has a significant effect on GLV production, with the highest effect for fungal treatments, followed by wounding and herbivory. **b**: Piercing/sucking herbivores release lower levels of GLVs compared to chewing herbivores. **c**: Treatment effects differ between monocotyledonous and eudicotyledonous plants, with a stronger effect in eudicotyledonous plants after fungal treatment, whereas monocotyledonous plants show a higher effect after herbivory. For easier interpretation of Figure 3-3b, two outliers with an effect size > 20 for the chewers are not shown, but can be seen in Fig. 3-3a, the number of studies used to calculate the effect is shown under the box plots. Statistical differences are calculated using one-way ANOVA with Welch correction and a post-hoc Dunnett T3 test for pairwise comparisons ($\alpha < 0.05$).

3.4.2 Herbivores can modulate the wound-induced release of GLVs

Plants respond to herbivory with the release of HIPVs amongst others which mainly consist of terpenoids, GLVs, aromatic compounds and amino acid derivatives (Clavijo McCormick *et al.*, 2012). While some of the HIPVs are only released upon herbivory and not by unwounded or mechanically wounded plants, others are released in higher quantities compared to wounded plants (Dicke & Baldwin, 2009). To distinguish folivory from pure mechanical damage, plants can recognize the frequency of damage (Mithöfer *et al.*, 2005) or the presence of HAMPs introduced into the wounds during feeding. Several of these HAMPs have been identified from the oral secretions (**OS**) of herbivores including fatty acid-amino acid conjugates (**FACs**, e.g. volicitin), sulfated fatty acids (caeliferins), and enzymes (e.g. β-glucosidase) (Acevedo *et al.*, 2015). Whereas induction of plant defenses is often accomplished via HAMPs, the suppression of induced or constitutive defenses is mainly attributed to so-called effector molecules (Kant *et al.*, 2015).

Our meta-analysis suggests that HAMPs do not play a major role in the GLV emission of plants, since the treatment effect for total GLV emission after herbivory is even lower than after mechanical damage (Figure 3-3). However, it is also possible that effectors are introduced during herbivory, which could mask a possible effect of HAMPs on GLV production. This yet lower treatment effect of herbivore infestation compared to mechanically wounded plants is quite unexpected, since it is commonly accepted that plants generally increase their GLV emission upon herbivory (Farag *et al.*, 2005; Shiojiri *et al.*, 2006a; Sufang *et al.*, 2013), and that this increase is merely due to the amount of physical damage that the attacker causes to the plant tissue. Since mechanical damage is quite often extensive and applied once while herbivory causes comparably little damage over a longer period of time, we cannot fully exclude the possibility that the observed differences in treatment effects, compared to the wounding treatment are simply a shortcoming of our meta-analysis. However, subdividing the herbivory treatment group into real herbivory and simulated herbivory (artificial wounding + OS) did not lead to a different outcome (Figure 3-8). Interestingly, only few studies exist that tried to disentangle the role of herbivore damage and salivary factors on the plant's GLV emission. These studies showed either amplification (Gaquerel *et al.*, 2009; Mattiacci *et al.*, 1995; Yan & Wang, 2006) or suppression (D'Auria *et al.*, 2007; Gaquerel *et al.*, 2009; Savchenko *et al.*, 2012; Allmann *et al.*, 2013) of the wound-induced GLV release. This suggests that the assumption – herbivory induced GLV emission results from the inflicted tissue damage during feeding - most likely derived from studies that used intact rather than wounded plants as control treatments.

Below, we elaborate on those studies reporting synergistic and antagonistic effects of salivary factors on GLV emission: application of β-glucosidase or OS from *Pieris brassicae*

larvae on wounded cabbage plants caused an increased emission of several GLVs, which exceeded the amounts released from purely mechanically damaged plants (Mattiacci *et al.*, 1995). Yan & Wang (2006) demonstrated that regurgitate of two different lepidopteran species, when applied to wounded maize seedlings, caused an amplification of the wound-induced release of Z-3-HAC. Other GLVs were not affected by the regurgitate-treatment. When OS of *Manduca sexta* larvae were applied to the wounds of *Nicotiana attenuata* leaves, these plants emitted higher levels of several Z-3-hexenyl esters (amongst others *Z*-3-hexenylpropionate, *Z*-3-hexenyl, *E*-2-butenoic ester and *Z*-3-hexenyltigilate) compared to wounded plants (Gaquerel *et al.*, 2009). Since these esters were hardly reported in other studies, probably due to their very low abundance, they were not included in our meta-analysis. Interestingly, the same study reports a decrease of the wound-induced release of *Z*-3-HOL when plants were treated with either *M. sexta* OS or a mix of FACs commonly present in *M. sexta's* OS.

Plants can evolve to recognize a pest and initiate direct and indirect defenses. This is subscribed by the fact that oral secretions of herbivores contain HAMPs which trigger e.g. the accumulation of certain phytohormones (Schmelz *et al.*, 2009) and induce the emission of certain HIPVs. It thus is not surprising that also herbivores can evolve to suppress these defenses. *Arabidopsis* plants which were attacked by *Spodoptera exigua* caterpillars released much smaller quantities of *Z*-3-HOL and *Z*-3-HAC compared to mechanically wounded plants (D'Auria *et al.*, 2007). Similar results were obtained when *Arabidopsis* leaves were treated with *P. rapae* OS (Savchenko *et al.*, 2012). This OS-induced suppression in the wound-induced GLV emission was regulated both transcriptionally and post-transcriptionally, and was restricted to the HPL branch of the oxylipin pathway; JA-levels were still upregulated in these plants upon OS-treatment (Savchenko *et al.*, 2012). In the wild tobacco plant, *N. attenuata,* the wound-induced increase in *HPL* transcript levels was suppressed by application of *M. sexta* OS onto wounded leaves. Surprisingly, this suppression was only detectable on the transcriptional and not on the metabolic level: GLV emission of OS-treated tobacco plants was even higher than that of wound-induced plants (Halitschke *et al.*, 2004b). The authors argued that this discrepancy - low *HPL* transcripts and high GLV emission - probably results from the fact that GLV biosynthesis is not controlled transcriptionally but rather by substrate flux, and that the release is limited by the supply of fatty acid 13-HPs that is readily metabolized by a high constitutive HPL activity that has been found in wild tobacco (Ziegler *et al.*, 2001). While evidence accumulates that the rate-limiting step indeed is upstream of HPL (Shiojiri *et al.*, 2006a; Hughes *et al.*, 2009), the timeframe that was chosen for this study to measure GLVs was too short to abandon the possibility that total GLV emissions might be suppressed by OS-application at a later time point; while volatiles were only measured within the first 20 min after treatment, the transcriptional analysis showed that

suppression of *HPL* started only 1 h after OS-treatment (Halitschke *et al.*, 2004b). Additionally, volatile measurements on wild tobacco plants that were taken 1-13h after OS-treatment did show that *Z*-3-HOL as well as 1-hexanol emissions were decreased compared to wounded plants (Gaquerel *et al.*, 2009). This is also apparent from our meta-analysis as we see a greater treatment effect at earlier time points for herbivores compared to wounding, but which attenuates at later time points (Figure 3-4). This response shows that the selection of the timeframe and the temporal resolution of the samples are important when performing a GLV analysis, as one could run the risk of missing or underestimating the plant response. Recent research reveals that also light and other (a)biotic factors play a role in the fine-tuning of the OS-induced modulation of GLV emission; in laboratory experiments total GLV emission of *Datura wrightii* plants treated with *M. sexta* OS did not differ from wounded plants during the day, but was clearly decreased during simulated sunset and night conditions. This OS-induced suppression in total GLV emission under laboratory low light conditions vanished when plants were grown in the field and thus encountered other (a)biotic stress factors as well (Allmann *et al.*, 2013).

These examples illustrate that the effect of herbivore OS on the wound-induced release of GLVs clearly depends on many different factors.

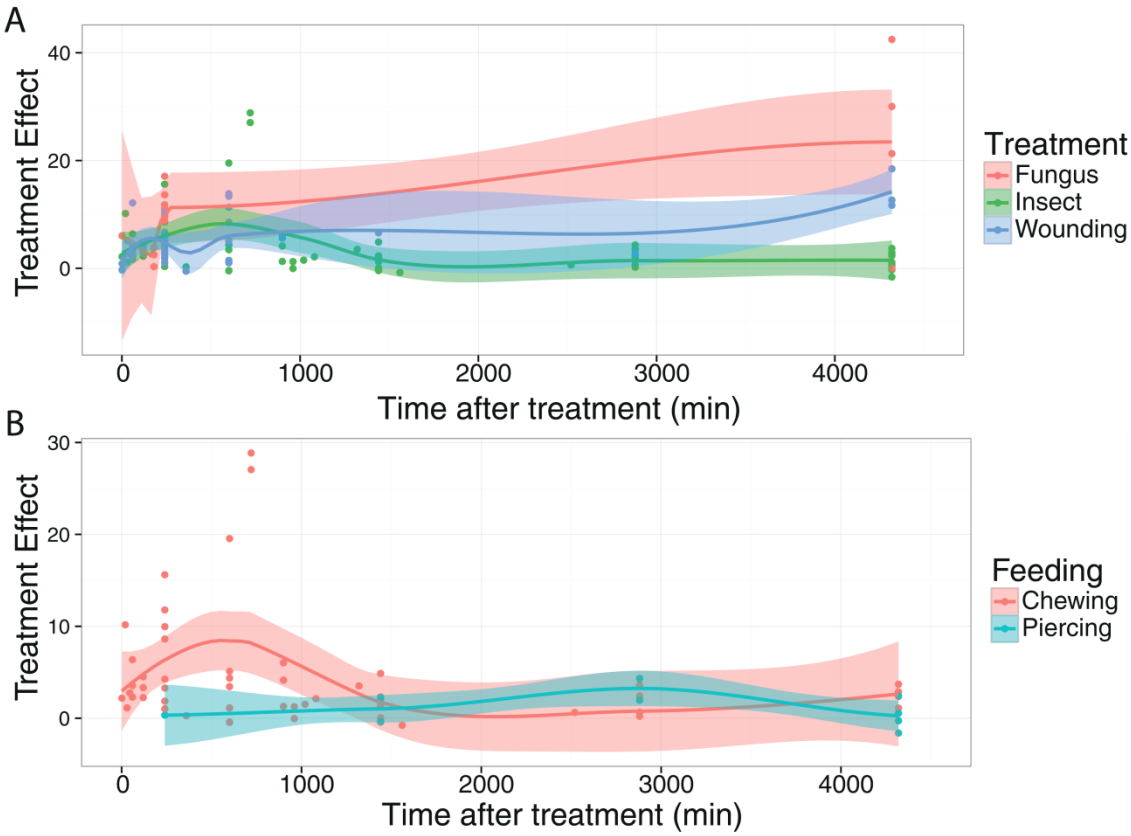

Figure 3-4: Time effect of different treatments on GLV production. A: Treatment effects at early time points are similar across the different treatments. However, at later time points, fungal treatments induce a greater effect on GLV production compared to the other treatments. Additionally, insects induce the lowest effect on GLV production. **B**: Within the insect treatment, insects with a piercing/sucking mode do not or only slightly induce GLV production at early time points, in contrast to chewing insects which induce a greater effect which levels off at

later time points. Lines represent locally weighted polynomial regression (LOESS); shaded areas represent the 95% confidence interval of the LOESS line.

Suppression of direct and indirect defenses has been shown for chewing as well as for piercing/sucking herbivores, such as mites, aphids and whiteflies (Kant *et al.*, 2015). Additionally, our meta-analysis demonstrated a difference in treatment effect depending on the feeding mode of the herbivore; while the treatment effect of chewing insects was 2.1 ($P < 0.001$) piercing/sucking herbivores showed a much smaller effect (TE = 0.36, $P < 0.05$) (Figure 3-3b). The lower GLV production upon infestation by piercing insects compared to chewing insects was consistent throughout a bigger time frame (Figure 3-4).

This difference in the treatment effect between the two feeding guilds, as described in our meta-analysis, might be attributed to differences in their ability to suppress or avoid recognition and defenses. However, the most obvious explanation is that piercing/sucking herbivores simply cause less damage to the plant thereby avoiding an increased release of GLVs. It is also possible that the difference in total GLV emission is caused by the activation of different defense pathways employed by plants. In general, but with an increasing number of exceptions (Kant *et al.*, 2015), piercing/sucking herbivores induce the SA dependent pathway, which can have an antagonizing effect on JA-induced defenses, whereas chewing herbivores mainly activate the JA-dependent pathway and subsequent defenses (Zarate *et al.*, 2007; Kant *et al.*, 2015). Because an increase in GLV emission often coincides with a higher JA-associated defense (Scala *et al.*, 2013a), the induction of JA-mediated defenses upon herbivore attack by chewers might explain the increased production of GLVs. Interestingly, tobacco plants with reduced transcript levels of Na*LOX3* – the LOX that specifically supplies substrate to the JA pathway, produced not only lower amounts of JA compared to WT plants but had also reduced *HPL* transcript levels (Halitschke & Baldwin, 2003; Allmann *et al.*, 2010). However, our meta-analysis does not answer whether piercing/sucking herbivores are better at suppressing GLV emissions, either directly, or indirectly by inducing hormonal defense pathways that do not influence GLV biosynthesis, or whether they simply cause less damage to the plant with their feeding style and thus induce lower levels of GLVs compared to chewers. Recently, a meta-analysis performed by Rowen & Kaplan (2016) also found a smaller treatment effect on GLV production for piercing/sucking herbivores, compared to chewers, which they also attributed to differences in induced defense pathways, instead of differences in cellular damage caused by feeding.

There are only very few studies that directly compare the role of different feeding guilds in inducing GLV emissions; in *Vicia faba* plants, pea aphid feeding did not induce GLV emission, but when plants were co-infested with *Spodoptera exigua* larvae*,* volatile emissions, including Z-3-HOL and Z-3-HAC, were decreased compared to those plants that

had been infested with caterpillars only (Schwartzberg *et al.*, 2011). This indicates that aphid derived effector molecules play a role in suppressing herbivory induced GLV emissions.

In the case of wound-induced GLV suppression by herbivores, it remains an open question whether it is the plant that evolved to recognize HAMPs or whether it is the herbivore that evolved to produce effectors in order to actively suppress wound-induced GLV emissions. From the plants perspective, GLVs have been shown to increase the attractiveness of plants to herbivores (Halitschke *et al.*, 2004b; Han *et al.*, 2012) and 'getting off the radar' (Halitschke *et al.*, 2008) could provide a level of protection for the plant. Additionally, GLVs have been shown to serve as feeding stimulant by increasing the consumption rate of lepidopteran caterpillars, and decreasing the amount of GLVs might be a good strategy to delay larval growth (Halitschke *et al.*, 2004b). On the other hand, GLVs are also known to attract predators and parasitoids to the herbivore infested plants (Shiojiri *et al.*, 2006b; Allmann & Baldwin, 2010; Shimoda, 2010) and 'getting off the radar' could provide a level of protection for the herbivore as well. In tomato, glycosylation of exogenous Z-3-HOL to Z-3-hexenylvicianoside had a direct toxic effect on the common cutworm *Spodoptera litura* (Sugimoto *et al.*, 2014). Thus, suppression of GLV production might be a measure for herbivores to reduce the amount of toxic GLV-metabolites.

3.4.3 Fungal infection greatly induces GLV production

While it is clear that GLVs mediate plant-insect interactions and that both plants and insects can benefit from altering the GLV production, it is more enigmatic how fungi may benefit from inducing GLV production. The meta-analysis showed that fungal infection caused the highest treatment effect compared to wounding and herbivory (Figure 3-3a). It remains unclear whether the high GLV production is an active response by the plant following recognition of fungal MAMPs, or a strategy by the pathogen to induce GLV production to promote virulence or whether GLV production is merely a by-product of cellular oxidative damage. In addition, the lifestyle of the fungus most likely also influences GLV production. Whereas biotrophic fungi thrive on living cells, necrotrophic fungi kill the host cell, thereby causing more cellular damage, which also may result in increased GLV production.

Unlike herbivores, fungal pathogens are restricted within the plant tissue, forcing them to employ sophisticated mechanisms to thrive on these plant cells. Production of phytotoxins (Möbius & Hertweck, 2009) or hijacking the plant hormone defense network are known strategies to successfully infect plant tissue (Maor & Shirasu, 2005; Yan & Xie, 2015). Some of these toxins are known to cause oxidative stress within the cell, which may lead to membrane damage resulting in substrate availability for GLV biosynthesis (Möbius & Hertweck, 2009). However, the significant larger effect compared to wounding suggests that

substrate availability does not fully account for the increase in GLV production after infection. It thus seems more likely that fungal effectors are involved in the increased GLV emission. To our knowledge, no fungal effectors have been described which attenuate or enhance GLV production. Notwithstanding, Blümke *et al.* (2014) showed that the pathogen *Fusarium graminearum* releases the effector lipase FGL1 to inhibit callose deposition in spikelets of wheat, thus enabling further colonization. The Δfgl1 mutant showed, together with reduced virulence, increased callose depositions and reduced levels of the free FAs linoleic acid and ALA. In addition, exogenously applied free FA restored the virulence of Δfgl1 to WT levels, showing that free FAs have an important role in the suppression of the innate immunity related to callose biosynthesis. Thus, it is tempting to speculate that FGL1 can increase the availability of free FAs, which could provide more substrate for GLV biosynthesis (Figure 3-2). However, recently a link between GLVs and the release of free FA was found; Li *et al.* (2016), reported a transient increase in ALA acid after treating *Z. mays* and *Solanum lycopersicum* with Z-3-HOL. They also found evidence for a priming effect of free FA for enhanced JA accumulation in distal plant tissue which could not be attributed to the increased substrate availability of ALA for JA biosynthesis. Together, these papers provide an indication that fungi could interfere with primary plant defenses (callose deposition) by influencing GLV biosynthesis and the subsequent free FA release.

The high GLV production after fungal infection can also be attributed to the plant hormonal defense response. Similar to insect infestations, fungal infection induces changes in defense hormone levels. Because of the many antagonistic and synergistic signaling pathways between different defense hormones, this leads to a whole rewiring of plant hormone network, allowing the plant to finely regulate its defenses to fend off fungal infection. (Pieterse *et al.*, 2012). Our meta-analysis predominantly contains studies on necrotrophic fungi (e.g. *Fusarium* spp., *Botrytis cinerea*), which have been shown to increase JA-levels in plants after infection (Ding *et al.*, 2011; El Oirdi *et al.*, 2011). The interaction between JA and GLVs has already numerously been reported to mutually induce each other, (Kessler & Baldwin, 2001; Engelberth *et al.*, 2004; Bruinsma *et al.*, 2009; Wei *et al.*, 2011; Engelberth *et al.*, 2013), which may account for the high induction of GLVs after fungal infection. It would be interesting to investigate the effect of biotrophic fungi on GLV production. Biotrophs might benefit more from a reduction in GLVs compared to necrotrophs. Additionally, research including biotrophs might shed light on the question whether the GLV induction is primarily MAMP- or effector driven or whether the amount of damage caused by biotrophic or necrotrophic fungi is the main driver of GLV production.

An active role for fungi in inducing GLV production is somewhat counterintuitive, because GLVs function as defensive compounds by exerting a direct negative effect against pathogenic fungi or by inducing and priming plant defenses (Kishimoto *et al.*, 2005;

Kishimoto *et al.*, 2006b; Ameye *et al.*, 2015). For example, Kishimoto *et al.* (2006a) found that exposing *A. thaliana* to the GLVs E-2-HAL and Z-3-HAL enhanced resistance against the necrotrophic fungus *Botrytis cinerea*. They accorded this enhanced resistance to increased lignification and higher transcripts of PDF1.2 and PR3, a chitinase. Further research by the same group additionally revealed that aforementioned GLVs possess fungicidal activity by inhibiting conidia germination and hyphae growth (Kishimoto *et al.*, 2008). The antifungal activity for C_6 aldehydes has also been shown against other fungi such as *Alternaria alternata*, *B. cinerea* (Hamilton-Kemp *et al.*, 1992) and *Aspergillus flavus* (De Lucca *et al.*, 2011). On the other hand, fungi might induce GLV production to influence another trophic level. As previously mentioned, insects can utilize GLVs to locate host plants. Induction of GLV production by fungi might benefit fungal infection and conidia dispersion by attracting herbivores which serve as vectors (Kluth *et al.*, 2002). Indeed, GLVs produced by infected plants are known to attract aphids (Webster & Cardé, 2016), which have recently been shown to promote fungal disease in wheat (Drakulic *et al.*, 2015; Drakulic *et al.*, 2016; De Zutter *et al.*, 2017).

However, fungi have often been shown to exploit the antagonistic signaling between defense hormones and as such hijack the plant defense machinery, in other words, fungi do not suppress plant defenses but manipulate the plant to invest in defense responses which are not effective to the pathogen in play or which even induce susceptibility (Verhage *et al.*, 2010). This interference in the plants metabolism is known to take place at several levels comprising both primary and secondary metabolism (Walton, 1996; Möbius & Hertweck, 2009). Thus, an increased defense response and concomitant increased GLV release does not necessarily mean a successful control of the pathogen by the plant. Indeed, the outcome of the GLV production during fungal infection is probably the combined effect of plant defense and fungal interference with that defense. As every fungal pathogen has its own arsenal of effectors (e.g. toxins) each interfering with plant defense in a specific way, one might expect that the GLV release in these plant fungal interactions is very variable. Our meta-analysis nicely supports this hypothesis: the variation of the treatment effect after fungal infection is large compared to the treatment effect after insect infestation or wounding (Figure 3-3a). This high variability is consistent in a broader time frame (Figure 3-4). As this dataset (Table 3-8) solely contained end-point studies, we do not have any information on how and whether the GLV production would change at different time points during the plant-pathogen interaction. Analyzing GLV production using Proton Transfer Reaction - Mass Spectrometry could shed light on the underlying pattern.

Similar to herbivores, fungi are able to locate hosts using chemical cues; hyphae can orient their growth towards chemical gradients (Brand & Gow, 2009). Recent research showed that the soilborne fungus *Fusarium oxysporum* can quickly reorient its growth in response to

sugars, amino acids and root exudates (Turrà *et al.*, 2015). Because of the conserved nature of GLV production it may be possible that pathogenic fungi have evolved to utilize these signals to orient themselves away (e.g. biotrophs) or towards (e.g. necrotrophs) these volatile cues. Investigating the interaction between fungal chemotropism and plant derived stress signals such as GLVs provides an exciting field of research (Turrà *et al.*, 2016).

Until recently, GLVs have not been associated with fungal or bacterial infection. However, evidence is culminating that GLVs also play an important role in plant-pathogen interaction. Still, compared to plant-insect research, there remains a knowledge gap. In order to shed more light on the role of GLVs in plant-pathogen interactions, more research on drivers of GLV biosynthesis after infection is mandatory.

3.4.4 Monocots and Eudicots respond differentially upon different types of stress

Our meta-analysis demonstrated an interaction between the type of stress the plant undergoes and whether the plant was mono- or eudicotyledonous (Table 3-4). Remarkably, for infections with fungi, the treatment effect (i.e. the standardized effect of a certain treatment on GLV production) was higher in dicots, compared to monocots (24.10 versus 8.76, respectively, $P = 0.027$), whereas for infestations with herbivores, monocots showed a higher treatment effect compared to eudicots (3.61 versus 1.26, respectively, $P < 0.001$) (Figure 3-3c). No difference between monocots or eudicots was found in the wounding treatment. To our knowledge, this is the first time that this discrepancy is reported, leaving us to speculate on the underlying mechanism. The difference in plant response to biotic stress between monocots and eudicots has already been demonstrated for plant defense hormones. While in dicots, a dichotomous model between SA (resistance against biotrophic pathogens) and JA (resistance against necrotrophs and herbivores) exists, in the monocot crop rice, there are multiple cases in which JA provides resistance against both biotrophic and necrotrophic pathogens (De Vleesschauwer *et al.*, 2014). As there currently is an absence of knowledge on the mechanism by which GLVs are perceived by the cell and how the signal is transduced, we cannot pinpoint whether the difference between mono- and eudicots can be situated there or whether it lies further downstream. Another hypothesis is that different MAMPs and HAMPS (e.g. chitin for fungal infection and FACs for herbivores) induce different responses in monocots and eudicots. Another difference between eudicots and monocots lies in the substrate competition for JA and GLV biosynthesis. In eudicots, HPL and AOS colocalize within the chloroplast (Froehlich *et al.*, 2001; Halitschke *et al.*, 2004b; Farmaki *et al.*, 2007), leading to substrate competition for 13-HPs. However, in maize it has been shown that the JA producing LOX8 is localized in the chloroplast, while the GLV

producing LOX10 is localized to non-chloroplast organelles, thus avoiding substrate competition (Christensen *et al.*, 2013b). While this might be the case for maize, in rice, another monocot, LOX is localized in the chloroplast (Zhou *et al.*, 2009), and negative crosstalk between HPL and AOS has been reported (Liu *et al.*, 2012; Tong *et al.*, 2012). Thus, substrate competition does not seem to explain the difference between monocots and eudicots in general. Whether the difference in effect between mono- and dicots can be explained by differences in hormone signaling or that effectors or substrate competition play a role remains to be elucidated.

3.4.5 The type of stress does not influence the proportion of GLVs per chemical class

Our meta-analysis provides convincing proof that plants increasingly release GLVs in response to biotic attackers. To determine whether the type of stress additionally has an influence on the composition of GLVs released from the plant, we subdivided the volatiles into three groups according to their chemical classes: aldehydes, alcohols and acetates. The meta-analysis revealed that wounding, herbivory or fungal infection did not cause a change in the relative composition of GLVs, compared to the control treatments, with the exception of the aldehydes after fungal infection, which constitutes the first step of GLV biosynthesis (Table 3-1). The general absence of a shift in the composition is most likely due to the enzyme reaction kinetics of GLV biosynthesis; overexpression of a bell pepper *HPL* in *Arabidopsis* (Ecotype No-0) did not lead to a constitutive increase in GLV emission compared to WT plants, which indicates that the limiting factor at least under control conditions must be upstream of HPL (Shiojiri *et al.*, 2006a). Similar conclusions have been drawn by other researchers (Hughes *et al.*, 2009) some of which suggested that lipases (Matsui *et al.*, 2000) are the rate-limiting step for GLV production. The 13-HPs which are produced by LOX (Figure 3-2) are most likely immediately cleaved by a highly active HPL, as has been proposed for *N. attenuata* (Ziegler *et al.*, 2001). Wounded *Arabidopsis* leaves (No-0) produced mainly *Z*-3-HOL while the levels of *Z*-3- and *E*-2-HAL were comparable to intact leaves. This lack of an increase in aldehydes suggests that the turn-over rate and thus either the activity or abundance of ADH, AKR or ADR must be very high as well (Matsui *et al.*, 2012). The fast production (Turlings *et al.*, 1998) and subsequent conversion of *Z*-3-HAL to its alcohols and esters have been described in several studies (Fall *et al.*, 1999; D'Auria *et al.*, 2007; Maja *et al.*, 2014). In wounded aspen leaves Z-3-HAL production peaked 5 min after wounding, declined rapidly and coincided with an increase of hexenyl alcohols and acetates between which the ratios stayed constant (Fall *et al.*, 1999). Our meta-analysis

suggests that after an initial burst of hexenals, other GLVs are quickly formed and reach constant ratios regardless of the type of biotic stress.

The fact that GLVs are rapidly released from damaged plant tissue makes them an interesting group of semiochemicals which could provide immediate information about the exact location of an attacking herbivore. However, the question arises: is the information encoded in the GLV bouquet also reliable and specific enough for prey searching carnivores if the composition does not change, as suggested in our meta-analysis? Previous research has shown that quantitative changes in single GLV compounds or a set of GLVs can clearly influence the behavior of predators and parasitoids (Kessler & Baldwin, 2001; Shiojiri *et al.*, 2006a; Chehab *et al.*, 2008).

While a change in the total amount of GLVs with no change in the composition most likely encodes for an insect only limited information, i.e. the amount of feeding damage rather than the nature of the attacker or the host plant, those quantitative changes in the GLV bouquet do affect the ratio between GLVs and other plant volatiles. Since insects often rely on a whole volatile blend rather than on single compounds for host plant selection (Bruce & Pickett, 2011) these relative changes compared to other groups of plant volatiles can suffice for insects to make informed choices.

Table 3-1: The relative composition of GLVs produced in plants after different treatments. The meta-analysis showed no significant difference in GLV composition between treatments. To calculate relative compositions of GLVs, we converted data from the studies used in the meta-analysis (see section 3.9). Statistical differences were calculated using a paired t-test ($\alpha < 0.05$).

		Median (Q1-Q3 quantile)			
Treatment	**Group**	**Before treatment**	**After treatment**	**P**	**n**
Fungus	Aldehydes	0.45 (0.28-0.58)	0.51 (0.47-0.58)	0.006	22
	Alcohols	0.22 (0.17-0.40)	0.18 (0.15-0.33)	0.115	22
	Acetates	0.27 (0.19-0.31)	0.29 (0.13-0.34)	0.320	22
Insect	Aldehydes	0.19 (0.15-0.48)	0.38 (0.06-0.51)	0.375	25
	Alcohols	0.30 (0.16 -0.39)	0.20 (0.14-0.31)	0.109	25
	Acetates	0.39 (0.22-062)	0.47 (0.28-0.65)	0.347	25
Wounding	Aldehydes	0.45 (0.19-0.59)	0.49 (0.40-0.53)	0.960	12
	Alcohols	0.18 (0.12-0.48)	0.22 (0.13-0.33)	0.464	12
	Acetates	0.28 (0.24-0.34)	0.34 (0.26-0.41)	0.298	12
Chewing	Aldehydes	0.30 (0.160-0.51)	0.44 (0.19-0.53)	0.200	19
	Alcohols	0.27 (0.16-0.37)	0.20 (0.15-0.27)	0.187	19
	Acetates	0.32 (0.21-0.60)	0.42 (0.25-0.57)	0.646	19
Piercing	Aldehydes	0.16 (0.01-0.18)	0.12 (0.01-0.18)	0.706	6
	Alcohols	0.36 (0.24-0.42)	0.26 (0.18-0.32)	0.385	6
	Acetates	0.54 (0.39-0.75)	0.67 (0.62-0.69)	0.383	6

While the un-specificity in the GLV signal might be an impairment for a host-searching insect, a simple increase in total GLV emission upon e.g. damage should be sufficient to serve as a damage signal for the plant itself to either induce or prime defense responses (Duran-Flores & Heil, 2016).

3.4.6 The type of stress does influence the isomeric ratio within each chemical class

While at first glance it seems as if herbivore-specific information cannot be conferred via the release of GLVs, recent studies revealed that both, plants (Kunishima *et al.*, 2016) and insects (Allmann & Baldwin, 2010) are able to fine-tune the plant's GLV composition; in wild tobacco herbivory by *M. sexta* caterpillars or the application of their OS to leaf wounds increased the conversion from *Z*-3- to *E*-2-GLVs. This decrease in the *Z*-3-/*E*-2-ratio tripled the foraging efficiency of the generalist predator *Geocoris* spp. in nature (Allmann & Baldwin,

2010). In this specific case it is not the plant that is responsible for the change in the GLV profile, but an enzyme present in the OS of the caterpillar. However, also plants are able to convert *Z*-3-HAL to its *E*-2-isomer by themselves: (3*Z*):(2*E*)-enal isomerase (HI) activity has been detected in the crude extracts of some plant species (Phillips *et al.*, 1979; Takamura & Gardner, 1996; Noordermeer *et al.*, 1999) and its sequence has recently been identified from red bell pepper (Kunishima *et al.*, 2016).

To determine whether conversion from *Z*-3- to *E*-2-hexenal is a general phenomenon in plants and whether the type of stress has an influence on this conversion, we compared *Z*-3-/*E*-2-ratios of aldehydes and alcohols after fungal infection or herbivory. The wounding treatment and hexenyl acetates were not included in this analysis due to the low number of studies that reported both isomeric forms. Our meta-analysis revealed that *Z*-3-/*E*-2-ratios significantly increased after fungal treatment (i.e. low conversion to *E*-2-GLVS) while herbivory did not cause a change in the ratio compared to control treatments (i.e. high conversion to *E*-2-GLVs)(Figure 3-5) Plant HIs are generally highly expressed in ripe fruits (paprika and cucumber, Phillips *et al.*, 1979; Kunishima *et al.*, 2016) and potato sprouts but show rather low constitutive expression levels in leaves. Wound-induced *E*-2-hexenal emissions are increased during the night (De Moraes *et al.*, 2001; Allmann *et al.*, 2013) and mechanical damage increases (3*Z*):(2*E*)-enal isomerase transcript levels (Kunishima *et al.*, 2016) This shows that plant HIs can be upregulated under certain circumstances. However this is apparently not the case upon fungal infection (this meta-analysis). This is rather unexpected since E-2-HAL is well known for its antimicrobial properties and has been reported to be at least in some cases even more effective than Z-3-HAL (Kishimoto *et al.*, 2005; Prost *et al.*, 2005). Future research could reveal whether fungi are able to manipulate the plant to produce less E-2-HAL.

Herbivory on the other hand clearly increases the conversion from *Z*-3- to *E*-2-hexenal (Figure 3-5). However, whether this general conversion is mainly caused by wound-inducible plant HIs or by insect derived HIs introduced into the wound during feeding is still an unanswered question. While an increased conversion has not only been observed with *M. sexta* OS, but also with the OS of, or herbivory by other lepidopteran species (Turlings & Wäckers, 2004; Allmann & Baldwin, 2010), it still needs to be tested how widespread the occurrence of such HIs is in other herbivores.

The shift from Z-3- to E-2-GLVs seems to be an important way to bring specificity to the GLV blend. However, it is still not clear what the main function of this increased conversion is. What is the evolutionary origin of HIs? Why do plants as well as insects possess HI activity? This might not be an easy question to answer, since it has been shown that an increase in *E*-2-GLVs can be beneficial for both plants and insects by attracting natural enemies of the herbivores (Allmann & Baldwin, 2010) and by informing gravid female

moths about the appropriateness of the host plant (Allmann *et al.*, 2013). Clearly, an increase in *E*-2-GLVs or changes in the ratio between *Z*-3- and *E*-2-GLVs can be sensed by insects of different trophic levels. However there are other, not mutually exclusive, hypotheses why plants and insects might increase the conversion-rate from Z-3-HAL to E-2-HAL and below we will elaborate on these:

Firstly, caterpillars may actively convert Z-3-HAL to E-2-HAL to protect themselves from endogenous and exogenous stresses: E-2-HAL is a reactive electrophile species (RES; Alméras *et al.*, 2003) as it possesses an α,β-unsaturated carbonyl group which shows high reactivity with nucleophilic atoms and can cause protein carbonylation (Farmer & Davoine, 2007; Mueller & Berger, 2009). RES-mediated carbonylation often leads to the inactivation of proteins (Liebler, 2008), however, it can also result in the activation of Cap'n'collar (Cnc) proteins which play an important role in regulating cellular defenses against oxidative and electrophilic stress (Nguyen *et al.*, 2009). The most studied Cnc TF is the vertebrate homolog Nrf2 (Sykiotis & Bohmann, 2010) and an ortholog, called CncC, has been identified for invertebrates (Drosophila; Sykiotis & Bohmann (2008)). Further research will reveal whether plants have a similar system to cope with oxidative stress. Secondly, herbivores and plants may actively produce E-2-HAL to protect themselves from pathogen attack: GLVs and especially C6-aldehydes have antimicrobial properties against bacteria (Nakamura & Hatanaka, 2002) and fungi (Hamilton-Kemp *et al.*, 1992). Only few studies exist that directly compared the antimicrobial properties of Z-3-HAL and E-2-HAL. While in some cases the two alkenals did not differ in their bacteriostatic effect (Nakamura & Hatanaka, 2002) or their anti-fungal activity (Tajul *et al.*, 2012), others reported higher antimicrobial activities of E-2-HAL compared to Z-3-HAL (Kishimoto *et al.*, 2005; Prost *et al.*, 2005).

Thirdly, plants may actively produce E-2-HAL to induce or prime plant defenses in neighboring plants or adjacent leaves of the same plant. This has been described for several GLVs (see next paragraph) but only few studies directly compared the efficiency of *Z*-3- and *E*-2-GLVs in inducing plant defense responses. While in maize seedlings *Z*-3-GLVs elicited a stronger response than *E*-2-GLVs (Ruther & Fürstenau, 2005), tomato plants showed an opposite trend by releasing higher levels of monoterpenes when exposed to E-2-HAL compared to Z-3-HAL (Farag & Paré, 2002). In any case, E-2-HAL has been shown to serve as a potent inducer of plant defenses (Zeringue Jr, 1992; Bate & Rothstein, 1998; Kessler *et al.*, 2006) and several E-2-HAL-specific marker genes have recently been identified in *Arabidopsis* (Mirabella *et al.*, 2015). Clearly, much more work is needed to understand whether compositional changes in the GLV bouquet are recognized by plants and used to modulate the induction or priming of plant defenses.

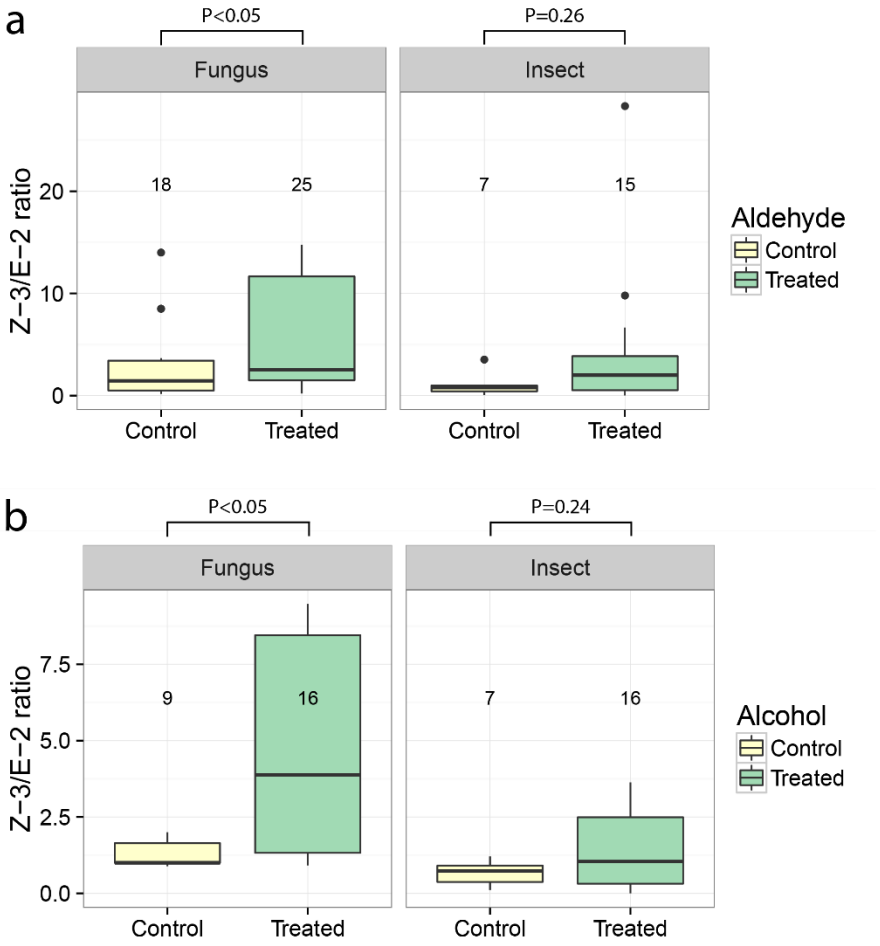

Figure 3-5: (Z)-3 to (E)-2 ratio of GLV isomers of aldehydes (a) and alcohols (b). The meta-analysis revealed an increase in the (Z-3)/(E-2)-ratio after fungal treatments, whereas the (Z-3)/(E-2)-ratio after herbivory did not significantly differ compared to intact plants. Statistical significance is calculated using one-way ANOVA with Welch correction and a post-hoc Dunnett T3 test (α<0.05)

3.5 GLVs: From signal perception to signal transduction

Volatiles can be used by insects to make informed choices about mating partners, habitats and appropriate host plants as food source or oviposition site (Dicke & Baldwin, 2009; Gadenne *et al.*, 2016), but also by plants to induce or prime defense responses (Kant *et al.*, 2009). Whereas the effect of GLVs on their interactors has been discussed within this review, this section will specifically focus on the functionality of GLVs for plants and discuss the recent knowledge of GLV perception and transduction in plants.

The idea that signaling between plants might be mediated by volatiles to activate defense responses emerged in the early eighties (Baldwin & Schultz, 1983; Rhoades David, 1983). Although controversial at that time, many studies have followed since, confirming that volatiles can initiate plant defense responses (Kant *et al.*, 2009; Karban *et al.*, 2014). Treating plants with GLVs can induce the expression of several defense-related genes and downstream metabolites (Zeringue Jr, 1992; Bate & Rothstein, 1998; Engelberth *et al.*, 2013)

including a subsequent release of BVOCs (Farag & Paré, 2002) or the secretion of extrafloral nectar (Kost & Heil, 2006). However, plants do not always directly up-regulate their defenses when exposed to plant volatiles. They can also be alerted by these volatiles, enabling them to induce their defenses more rapidly and/or more effectively at the actual time of herbivore or pathogen attack. This form of alertness is called priming and it has the advantage that it does not involve a significant fitness penalty for the plant (Martinez-Medina *et al.*, 2016). A pioneering study by Engelberth *et al.* (2004) showed that exposure of maize plants to pure GLVs increased JA production and the release of several terpenoids after challenge with crude regurgitant from *Spodoptera exigua*. Since then, several other studies have followed and confirmed the priming effect of GLVs on plants upon herbivory (Kost & Heil, 2006; Engelberth *et al.*, 2007; Frost *et al.*, 2008b; Engelberth *et al.*, 2013; Li *et al.*, 2016). Recently, the priming potential of GLVs has also been shown to act against pathogens. Exposing wheat to Z-3-HAC primed plants for enhanced defense against the pathogenic fungi *Fusarium graminearum* by increasing JA dependent signaling (Ameye *et al.*, 2015). Even though in the latter study, wheat seedlings were exposed to a high concentration of Z-3-HAC (0.11 mM), aerial Z-3-HAC concentration declined very rapidly to previously reported concentrations (Section 4.8) (Piesik *et al.*, 2011; Wenda-Piesik *et al.*, 2010). As stated by Matsui *et al.* (2012), aqueous concentrations of GLVs within cells can reach values up to 1 mM. Thus, using a high concentration for a short period of time may actually mimic GLV concentrations in damaged cells. Plants can eavesdrop on the volatile signals coming from their neighbors, but plant signaling by GLVs is more likely to occur within one plant and might serve to overcome vascular constraints or to augment vascular systemic signals (Heil & Ton, 2008). In the case of intra-plant signaling GLVs should be defined as DAMPs as proposed by Duran-Flores & Heil (2016).

The observation that plants can use volatile information coming from neighboring plants or adjacent leaves raises the question about the perception and signal transduction of GLVs. Whereas in recent years an increasing amount of knowledge has been collected about the molecular and cellular processes of odorant detection in insect antennae and the primary processing of olfactory signals in the brain (Carey & Carlson, 2011), the knowledge about volatile perception by plants is still in its infancy (Heil, 2014) and a volatile receptor has so far only been identified for ethylene (Schaller & Bleecker, 1995). In insects, receptor proteins are key elements for the recognition and discrimination of plant volatiles (Leal, 2013); different types of GLVs can cause various types of responses in insects, both, at the physiological (Hansson *et al.*, 1999; Røstelien *et al.*, 2005; Allmann *et al.*, 2013) and the behavioral level (Reinecke *et al.*, 2005; Allmann *et al.*, 2013). In plants, such specificity has been shown in some but not all cases: while structurally different GLVs (Z-3-HOL, Z-3-HAL and E-2-HAL) can have different efficacies in activating monoterpene emission in tomato (Farag & Paré,

2002) neither configuration nor the position of the double bond of GLVs were important for the induction of extrafloral nectar in lima beans (Heil *et al.*, 2008).

In order to transfer GLV-encoded information into plant cells GLVs need to reach the plasma membrane or other organelles of the plant cell, either via active or passive transport. Plant volatiles are most likely taken up by the plant via their stomata or by adsorption through the leaf surface (Matsui, 2016). Still, before reaching the plasma membrane they have to cross the cuticle and the cell wall. How this occurs is still unknown but due to their lipophilic character, GLVs can ”dissolve” in the plasma membrane as suggested by Heil (2014) but they can also reach the cytosol where they are further metabolized by the plant cell (Figure 3-6) (Fig. 6.; Farag & Paré, 2002; Matsui *et al.*, 2012). Reported metabolization processes of GLVs include glutathionylation of E-2-HAL (Figure 3-6, IV) (Fig. 6, IV; Davoine *et al.*, 2006; Mirabella *et al.*, 2008) and the glycosylation of Z-3-HOL (Figure 3-6, III)(Sugimoto *et al.*, 2014). Early events in leaves upon GLV exposure include plasma membrane potential ($\mathbf{V_m}$) depolarization and an increase in $[Ca^{2+}]_{cyt}$. Exposure of tomato plants to several GLVs triggered within seconds a depolarization of mesophyll cells (Figure 3-6, 1) and within minutes an increase in cytosolic calcium $[Ca^{2+}]_{Cyt}$ (Zebelo *et al.*, 2012). Similar results have been found in *Arabidopsis*; exposure of leaves to E-2-HAL and E-2-HOL induced a fast and transient increase in $[Ca^{2+}]_{Cyt.}$ (Asai *et al.*, 2009). This increase can be caused by the activation of a depolarization-activated calcium channel (**DACC**) (Figure 3-6, 2a), a ROS-activated calcium channel (Figure 3-6, 2b) or via the release of Ca^{2+} from vacuoles (Figure 3-6, 2c) (Swarbreck *et al.*, 2013). While activation of DACCs is most likely triggered by many if not all GLVs (Zebelo *et al.*, 2012), an increase in $[Ca^{2+}]_{cyt}$ via ROS-activated calcium channels seems to be more specific for reactive electrophilic species (**RES**)-type GLVs, like E-2-HAL (Asai *et al.*, 2009).

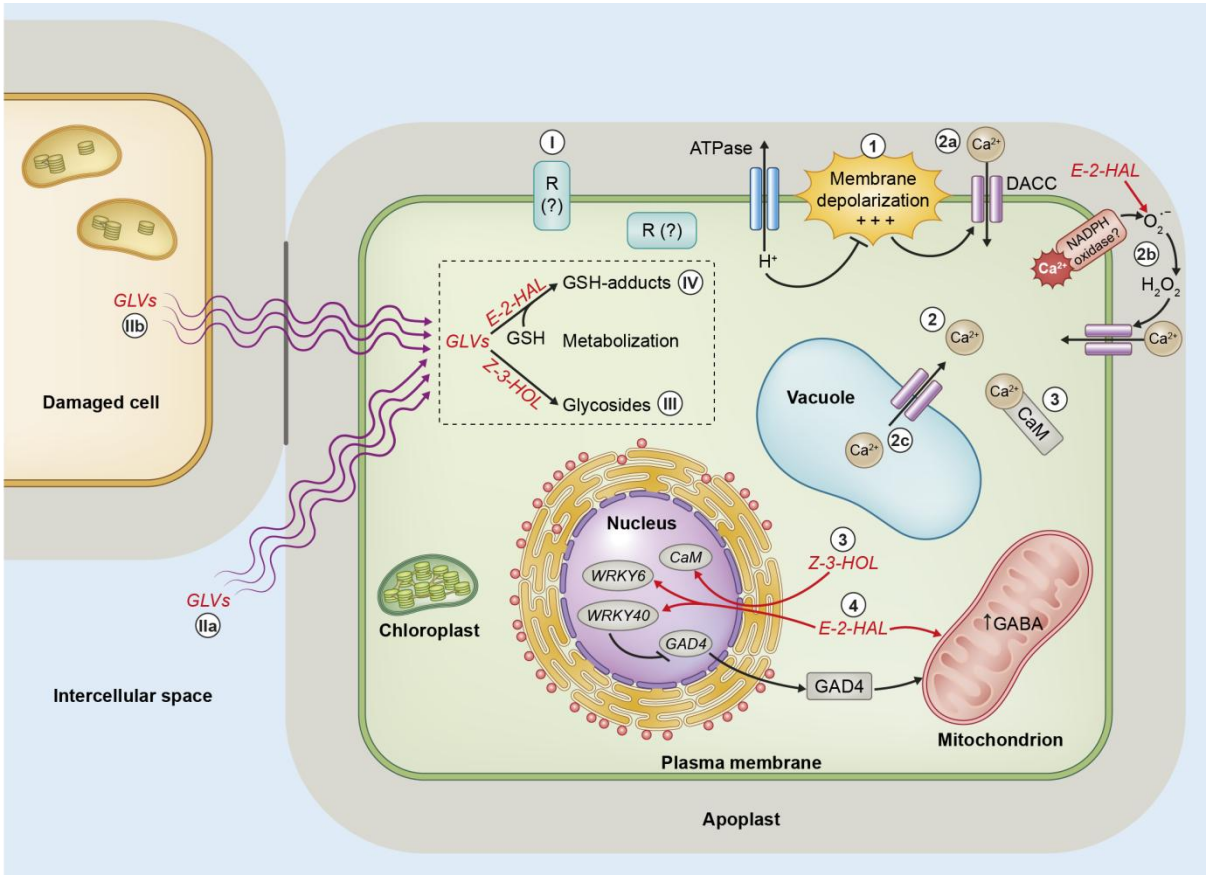

Figure 3-6: Signal transduction by green leaf volatiles. Early events in leaves upon GLV exposure include (1) plasma membrane potential (V_m) depolarization (tomato), and (2) an increase in $[Ca^{2+}]_{cyt}$ (Arabidopsis and tomato). The increase in $[Ca^{2+}]_{cyt}$ can be caused by the activation of (2a) a depolarization-activated calcium channel (DACC), (2b) a ROS-activated calcium channel or (2c) via the release of Ca^{2+} from vacuoles. Exposure of maize leaves to Z-3-HOL induces (3) the transcript levels of Calmodulin (CaM). Other early signaling responses include (4) the transcriptional activation of WRKY 40 and 6 by E-2-HALin *Arabidopsis*. These transcription factors negatively regulate the transcription of GAD4.While it has been assumed that plants can recognize GLVs by specific protein receptors (R) they have not been identified yet (I). GLVs can at least partly enter the plant cell passively (II). These GLVs either origin from neighboring leaves or plants and enter the plant most likely via the stomata (IIa), or are produced in damaged cells to reach intact neighboring cells (IIb). GLVs that enter the cell can be metabolized in the cytoplasm by glycosylation (III) or glutathionylation (IV). The middle lamella is only indicated as a black line between the two plant cells. GLVs, green leaf volatiles; R, receptor; *E*-2-HAL, *E*-2-hexenal; *Z*-3-HOL, *Z*-3-hexenol; GSH, glutathione.

Some of the downstream molecular players in GLV signaling have recently been elucidated; exposure of maize leaves to Z-3-HOL for either 20 or 60 min increased the transcript levels of several genes involved in signaling, including transcription factors, genes related to phosphorylation, calcium (Figure 3-6, 3) and lipid signaling. While transcriptional regulators accounted for almost 50% of the differentially regulated genes 20 min after GLV exposure they represented only a minor group of 10-15% after 60 min. Interestingly, there was hardly any overlap in regulated genes between early and late time points (Engelberth *et al.*, 2013). Other early signaling responses include the transcriptional activation of *WRKY40* and *6* by E-2-HAL in Arabidopsis (Figure 3-6, 4)(Mirabella *et al.*, 2008; Mirabella *et al.*, 2015). Early signaling responses do not only include transcriptional, but also metabolic changes; an increased release of free FAs within 15 min of exposure to Z-3-HOL has recently been reported to be a common feature of several plant species, including monocots and dicots. In

maize seedlings this increase in free FAs seems to be involved in plant priming upon herbivory (Li *et al.*, 2016).

3.6 GLVs influence the C/N metabolism

In order to mount their defenses plants need to redistribute energy and metabolites towards the defense machinery (Berger *et al.*, 2007; Bolton, 2009). Glutamate plays an important role in this (Seifi *et al.*, 2013b): after pathogen attack, plants can maintain cell viability by maintaining glutamate levels in infected tissue and replenishment of the tricarboxylic acid (TCA) cycle through the GABA-shunt. This scenario is efficient when a plant is attacked by a necrotrophic pathogen. However, plants can also induce cell death by depleting glutamate levels and exhausting the TCA cycle. This strategy is used when a plant is under attack of a biotrophic pathogen. These strategies have been named endurance and evasion, respectively. The interference of pathogens with this C/N mechanism constitutes an important part of the infection strategy of several, often toxigenic fungal pathogens such as *Fusarium graminearum* (Audenaert *et al.*, 2013)*, Alternaria spp.* (Klotz, 1988)*, Cochliobolus spp*. (Stergiopoulos *et al.*, 2013). Changes in the primary metabolism, including nitrogen assimilation, are also a well-known phenomenon upon insect herbivory (Zhou *et al.*, 2015); aphid attack induced the expression of a nitrite reductase in sorghum (Zhu-Salzman *et al.*, 2004) and a glutamate synthase (GOGAT) in *N. attenuata* (Voelckel *et al.*, 2004). Both enzymes are required for the nitrogen assimilation into glutamate.

No link between C/N metabolism and GLVs was expected until recent work on Arabidopsis revealed upregulation of *GAD4*, a key enzyme in the GABA shunt, after plants were exposed to E-2-HAL, coinciding with an increase in GABA levels (Mirabella *et al.*, 2008; Mirabella *et al.*, 2015). Interestingly, the same study showed that E-2-HAL also induces expression of the transcription factor WRKY40, which negatively regulates *GAD4* expression. Microarray data from maize plants which had been exposed to Z-3-HOL for 60 min show that transcript levels of glutamine-fructose-6-phosphate transaminase 2, which converts glutamine to glutamate, were significantly upregulated. This suggests that N is mobilized towards the exposed tissue under the form of glutamine, which is locally converted to glutamate (Engelberth *et al.*, 2013). Together, these data point to a model in which plant cells upon GLV exposure, aim to maintain cell viability through TCA replenishment and nitrogen remobilization. The importance of the TCA replenishment in mounting a successful defense remains enigmatic.

3.7 Interaction with plant hormones

GLVs have mostly been studied in the context of plant-insect interactions of which defense has generally been attributed to JA (Pieterse *et al.*, 2012). Consequently, most research on

GLVs focused on the effect of GLV on JA signaling. However, over the last decade it has become increasingly apparent that plant defense is regulated by a network of plant hormones, and that a complex grid of crosstalk between these hormonal pathways moulds the outcome of the plant-insect/pathogen interaction (De Vleesschauwer *et al.*, 2014; Nguyen *et al.*, 2016).

The interaction between GLVs and JA has often been reported (Halitschke *et al.*, 2004b). For example, treatment with GLVs resulted in increases in JA biosynthesis genes in *Arabidopsis* (Bate & Rothstein, 1998; Kishimoto *et al.*, 2005; Kishimoto *et al.*, 2006b), maize (Engelberth *et al.*, 2004), lima bean (Arimura *et al.*, 2001), citrus (Gomi *et al.*, 2003), and tea (Xin *et al.*, 2015) but not in tomato (Sugimoto *et al.*, 2014).

Furthermore, exogenously applied JA or GLVs induce Z-3-HOL, E-2-HAL and Z-3-HAC production in tomato plants and cereals (Wei *et al.*, 2011; Piesik *et al.*, 2013), creating a positive feedback loop. This mechanism allows the plant to warn plants of an impending fungus/insect attack and induce or prime defenses in systemic tissue as well as in neighboring plants.

Wei *et al.* (2011) showed that *35S::prosys* tomato plants with constitutive JA signaling constitutively released Z-3-HOL, even in the absence of damage by insect herbivores. They hypothesized that overexpressing the prosystemin gene enhances expression of LOX and HPL, which subsequently leads to higher GLV production. Tomato mutants defective in JA biosynthesis (*spr2*) did not abolish Z-3-HOL production after *S. exigua* damage, which suggests that GLV production is not exclusively governed by JA signaling. Bate & Rothstein (1998) found that treatment of *Arabidopsis* seedlings with E-2-HAL induced expression of genes involved in the oxylipin biosynthesis and phenylpropanoid pathway. However, no induction of pathogenesis related proteins was found. Interestingly, seed germination was reduced by E-2-HAL in the wild type as well as in the JA insensitive mutant (*jar1-1*) suggesting that E-2-HAL perception and signaling is independent of the JA pathway. In addition, *PR1* and *PR2* did not show an upregulation in response to GLV treatment, whereas MeJA induced expression of *PR1* and *PR*2 suggesting separate signaling pathways.

More recently, attention to the effect of GLVs on plant hormones has shifted from a focused view on JA biosynthesis and signaling to a more holistic approach using transcriptomics. Mirabella *et al.* (2015) found that gene activation by E-2-HAL in *Arabidopsis* was most closely related to gene activation after SA treatment (50%), followed by ABA (29%) and JA (13%), whereas ethylene treatment only showed a 1% overlap with E-2-HAL treatment. While these percentages give an idea on the overlap with other defense hormones, they do not give information on the signaling pathways which are activated. In other words, a 13% overlap may include all genes which play crucial roles in defense signaling, while the 50% might not all include important defense genes. While omics-studies in general provide an

unbiased look, further studies are needed to elucidate the underlying mechanism. The same study additionally reported that one third of the upregulated genes by E-2-HAL were unique for the E-2-HAL treatment and independent of aforementioned plant hormones. This confirmed earlier work from the same research group which exhibited an inhibitory effect of E-2-HAL on root growth of *Arabidopsis* which was independent of JA, SA, ABA and ethylene signaling (Mirabella *et al.*, 2008). This partly contradicts the study by Kishimoto *et al.* (2006b) which reported that the induction of defense genes in *Arabidopsis*, after treatment with E-2-HAL and Z-3-HAL was significantly repressed in JA and ethylene deficient signaling mutants, but did not have an effect in the SA insensitive mutant. However, in maize, Z-3-HOL induced expression of phenylalanine ammonia lyase, a gene in the biosynthesis of SA. As aforementioned, this opens up the question whether different GLVs induce different responses and whether knowledge gathered on *Arabidopsis* can be transferred to other crops.

In summary, GLVs have an inducing effect on JA biosynthesis and JA dependent signaling. However, evidence provided by different studies suggests that GLV signaling is not exclusively governed by JA dependent signaling which might point to a separate, yet unknown signaling pathway. To obtain a better insight into the role of GLVs in the plant hormone network, more research on the effect of GLVs on the plant hormone (signaling) network is needed.

3.8 General conclusions

A meta-analysis approach offers a holistic view on GLV literature, and allows us to uncover yet unknown factors in the production of GLVs. However, the results must be carefully interpreted and conclusions drawn from this analysis cannot serve as *a one size fits all* for each plant-pathogen/herbivore interaction. Nonetheless, some surprising trends became apparent. The high induction of GLV production upon fungal infection, compared to herbivore treatment remained, until now, largely overlooked in literature, but opens up an exciting field of research. Evidence is culminating that herbivores modulate GLV emission. Besides suppression or enhancement of GLV production, herbivores can also increase the Z-3- to E-2-conversion. Furthermore, there was a clear discrepancy between monocots and eudicots which should be taken into account in further research and applications.

Despite the progress which has been made the last years in unraveling the functions and mechanisms underlying GLV perception and signaling, a lot of questions remain unanswered. Major questions are: How do plants perceive GLVs and via which mechanism is the signal transduced? Do GLVs solely interact with JA or do other plant hormones play a role? What is the underlying reason for the difference in GLV production between monocots and dicots? What is the ecological relevance of *Z*-3- to *E*-2-conversion of GLVs for plants

and insects? Can GLVs be used in agronomic practices; e.g. as alternatives for pesticides? We strongly believe that unraveling these questions is of paramount importance and will provide a deeper understanding about a trait which has been preserved throughout the plant kingdom and serves as a general stress signal against (a)biotic stress.

3.9 Addendum: Procedure for the meta-analysis

3.9.1 Data acquisition and selection for the meta-analysis

Publications were collected using the online search tools: Web of Science ™ and Google Scholar. A combination of the following keywords was used in the online search: plant, volatile, green leaf volatile, hexen*, headspace analysis, insect, wounding, damage, fungus, pathogen. Only studies were withheld which met following criteria:

- ≥3 replicates
- At least two GLVs are reported
- Control and treatment GLV measurements are reported
- Standard deviations or standard errors are reported
- Masses or molar concentrations are reported (no peak areas or peak intensities)

3.9.2 Studies included in the meta-analysis

Allmann S, Späthe A, Bisch-Knaden S, Kallenbach M, Reinecke A, Sachse S, Baldwin IT, Hansson BS. 2013. Feeding-induced rearrangement of green leaf volatiles reduces moth oviposition. *Elife* **2**: e00421.

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Blackmer J, Rodriguez-Saona C, Byers J, Shope K, Smith J. 2004. Behavioral response of *Lygus hesperus* to conspecifics and headspace volatiles of alfalfa in a Y-tube olfactometer. *Journal of Chemical Ecology* **30**: 1547-1564.

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Copolovici L, Kännaste A, Remmel T, Vislap V, Niinemets Ü. 2011. Volatile emissions from *Alnus glutionosa* induced by herbivory are quantitatively related to the extent of damage. *Journal of Chemical Ecology* **37**: 18-28.

D'Auria JC, Pichersky E, Schaub A, Hansel A, Gershenzon J. 2007. Characterization of a BAHD acyltransferase responsible for producing the green leaf volatile (Z)-3-hexen-1-yl acetate in *Arabidopsis thaliana*. *The Plant Journal* **49**: 194-207.

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Gossner MM, Weisser WW, Gershenzon J, Unsicker SB. 2014. Insect attraction to herbivore-induced beech volatiles under different forest management regimes. *Oecologia* **176**: 569-580.

Himanen S, Vuorinen T, Tuovinen T, Holopainen JK. 2005. Effects of cyclamen mite (*Phytonemus pallidus*) and leaf beetle (*Galerucella tenella*) damage on volatile emission from strawberry (*Fragaria*× *ananassa* Duch.) plants and orientation of predatory mites (*Neoseiulus cucumeris*, *N. californicus*, and *Euseius finlandicus*). *Journal of Agricultural and Food Chemistry* **53**: 8624-8630.

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Kariyat RR, Mauck KE, De Moraes CM, Stephenson AG, Mescher MC. 2012. Inbreeding alters volatile signaling phenotypes and influences tri-trophic interactions in horsenettle (*Solanum carolinense* L.). *Ecology Letters* **15**: 301-309.

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Khaling E, Li T, Holopainen JK, Blande JD. 2016. Elevated Ozone Modulates Herbivore-Induced Volatile Emissions of Brassica nigra and Alters a Tritrophic Interaction. *Journal of Chemical Ecology*: 1-14.

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McCall PJ, Turlings TC, Loughrin J, Proveaux AT, Tumlinson JH. 1994. Herbivore-induced volatile emissions from cotton (*Gossypium hirsutum* L.) seedlings. *Journal of Chemical Ecology* **20**: 3039-3050.

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Rodriguez-Saona C, Crafts-Brander SJ, Pare PW, Henneberry TJ. 2001. Exogenous methyl jasmonate induces volatile emissions in cotton plants. *Journal of Chemical Ecology* **27**: 679-695.

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Wei J, Wang L, Zhu J, Zhang S, Nandi OI, Kang L. 2007. Plants Attract Parasitic Wasps to Defend Themselves against Insect Pests by Releasing Hexenol. *Plos One* **2**.

Wenda-Piesik A. 2011. Volatile Organic Compound Emissions by Winter Wheat Plants (*Triticum aestivum* L.) under *Fusarium spp*. Infestation and Various Abiotic Conditions. *Polish Journal of Environmental Studies* **20**: 1335-1342.

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Zebelo SA, Matsui K, Ozawa R, Maffei ME. 2012. Plasma membrane potential depolarization and cytosolic calcium flux are early events involved in tomato (*Solanum lycopersicon*) plant-to-plant communication. *Plant Science* **196**: 93-100.

3.9.3 Treatment effect

The meta-analysis is largely based on the methodology used in Rowen and Kaplan (2016) with minor modifications. In short, the treatment effect was calculated using Hedges' g, which represents the standardized mean difference between a treatment and control (Hedges, 1981). The equation can be found below:

$$g = \frac{\overline{x_1} - \overline{x_2}}{s^*}$$

$$s^* = \sqrt{\frac{(n_1 - 1){s_1}^2 + (n_2 - 1){s_2}^2}{n_1 + n_2 - 2}}$$

Where, $\overline{x_1}$ and $\overline{x_2}$ represent the mean of the treatment and the control, respectively. s^* represents the pooled standard deviation of the treated and control means. n_1 and n_2 represent the number of replicates in the treated and control treatments, respectively and s_1 and s_2, represent the standard deviation of the treated and control treatments, respectively.

As variable, the sum of all green leaf volatiles was used. Calculations and plots were made using the packages "meta" and "ggplot2", respectively in R (build 3.2.4).

3.9.4 Publication bias

Funnel plots were created to verify the presence of potential publication bias (i.e. studies that report higher treatment effects are more likely to be published than studies with lower treatment effect) (Sterne and Egger, 2001). In the absence of publication bias, the studies will be distributed symmetrically around the mean treatment effect in a funnel plot. Asymmetry in funnel plots may indicate publication bias. Funnel plots were created using the package "meta" in R and are displayed in (Figure 3-7). Interpreting the funnel plots, we can see that for the "Fungus" and "Wounding" treatment, the funnel plots are asymmetrical and that a publication bias may be present.

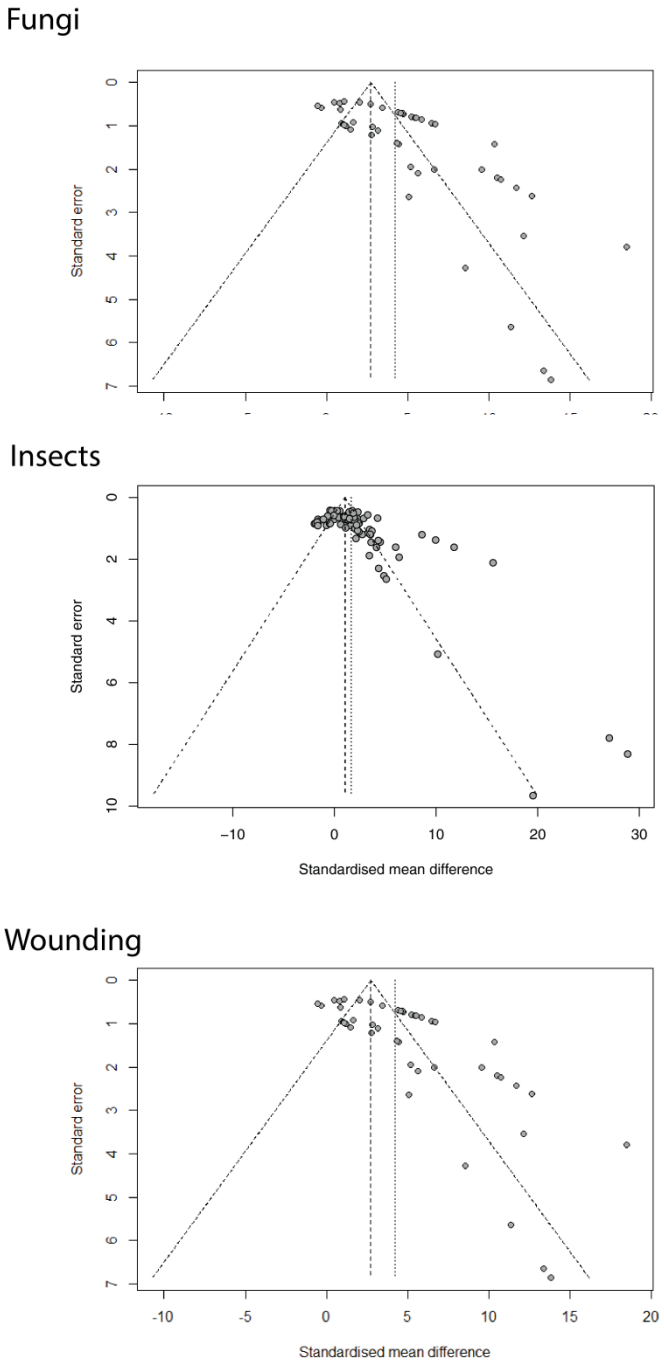

Figure 3-7: Funnel plots for total GLV production after treatment by a fungus, insect or wounding. Funnel plots depict the standardized mean difference (Treatment effect) on the x-axis and the standard error of the treatment effect on the y-axis. Each point represents an experiment of the studies included in the meta-analysis. If no publication bias is present, studies will cluster symmetrically around the mean treatment effect.

3.9.5 Robustness of the data

To ascertain whether the reported treatment effect is robust and cannot be attributed to the potential publication bias, we calculated the fail-safe number, using the Rosenberg method (Table 3-2)(Rosenberg, 2005). The fail-safe number represents the amount of cases with a nonsignificant treatment effect that should be added to the meta-analysis to obtain a non-

significant treatment effect. The fail-safe number for each treatment is calculated using the package “meta” in R.

Table 3-2: Fail-safe number of the different treatments.

Treatment	Fail-safe number (Rosenberg approach)	Number of studies included in the analysis
Fungus	1856	26
Insect	3847	88
Wounding	6099	48

Hedges LV (1981) Distribution theory for Glass's estimator of effect size and related estimators. Journal of Educational and Behavioral Statistics **6:** 107-128

Rosenberg MS (2005) The file-drawer problem revisited: a general weighted method for calculating fail-safe numbers in meta-analysis. Evolution **59:** 464-468

Rowen E, Kaplan I (2016) Eco-evolutionary factors drive induced plant volatiles: a meta-analysis. New Phytologist

Sterne JA, Egger M (2001) Funnel plots for detecting bias in meta-analysis: guidelines on choice of axis. Journal of clinical epidemiology **54:** 1046-1055

3.9.6 Statistical values for the meta-analysis

Table 3-3: Treatment effect and one sample, two sided t-test (H0: Treatment effect=0) for the different treatments.

Treatment	Treatment effect (mean)	Treatment effect (median)	n	t-value	P-value
Fungus	12.19	9.67	26	5.744	<0.001
Insect	2.88	1.86	88	5.207	<0.001
Wounding	5.47	4.69	48	8.676	<0.001
Chewing	3.30	2.10	64	4.265	<0.001
Piercing	0.83	0.36	23	2.722	0.012

Table 3-4: Two-way ANOVA on the treatment effect with Treatment (Fungus, Insect, Wounding) and Taxa (monocots, eudicots) as fixed factors

Source	Type III sum of squares	df	Mean Square	F	P-value
Treatment	2611.276	2	1282.488	45.917	<0.001
Taxa	754.924	1	779.856	27.921	<0.001
Treatment*Taxa	1712.171	2	836.906	29.964	<0.001
Error	4711.753	156	27.931		
Corrected Total	8382.494	161			

3.9.7 Treatment effect of real herbivory vs. oral secretions

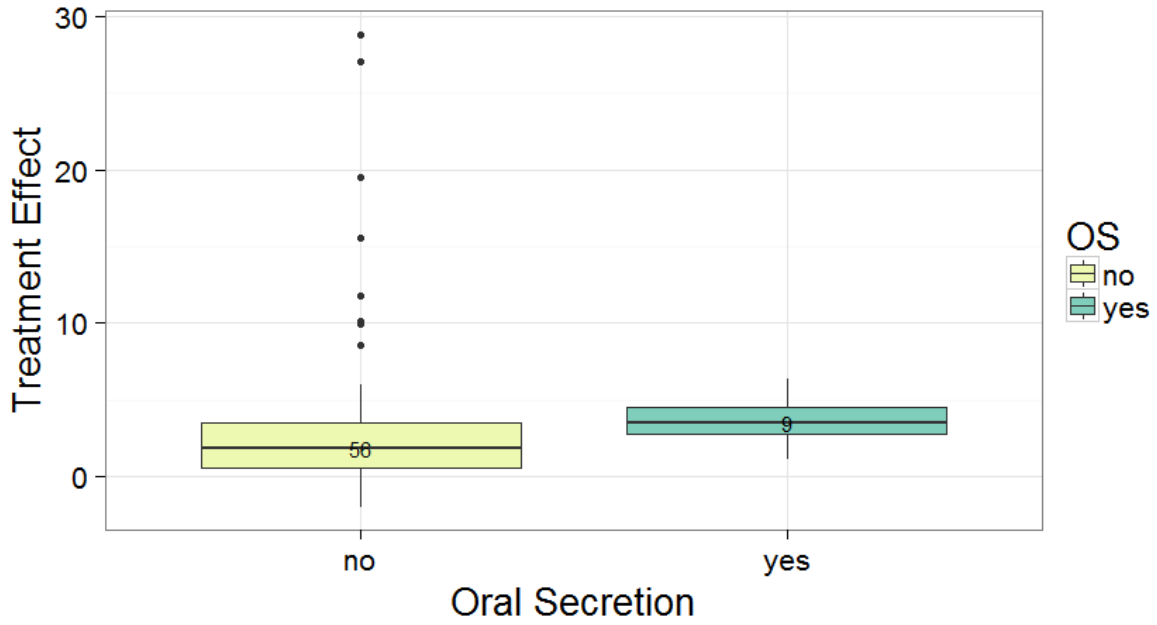

Figure 3-8: Treatment effects are not significantly different (P>0.05) between studies which use simulated or real herbivory. The number of studies used to calculate the effect is shown inside the boxplots. Significant differences were calculated using Student's t-test. Simulated herbivory, wounding + OS (OS, yes); real herbivory (OS, no).

3.9.8 Datasets

Table 3-5: Insect treatment dataset derived from the meta-analysis using Hedges' g to calculate the treatment effect (TE). Abbreviations: OS, oral secretions; TE, treatment effect. NA: not available.

Study	Plant	Plant Family	Eudicot/ Monocot	Insect	Feeding mode	Volume OS (µl)	# Insects	Minutes after treatment	TE
Allmann et al. (2013)	*Datura wrightii*	*Solanaceae*	Eudicots	*Manduca sexta*	Chewing	6.66		120	4.508391
Allmann et al. (2013)	*Datura wrightii*	*Solanaceae*	Eudicots	*Manduca sexta*	Chewing	6.66		120	3.329119
Allmann et al. (2013)	*Datura wrightii*	*Solanaceae*	Eudicots	*Manduca sexta*	Chewing	6.66		120	2.238967
Blackmer et al. (2004)	*Medicago sativa*	*Fabaceae*	Eudicots	*Lygus hesperus*	Piercing		30	240	0.329361
Blande et al. (2010)	*Betula pendula*	*Betulaceae*	Eudicots	*Euceraphis betulae*	Piercing		100	5760	-0.66549
Blande et al. (2010)	*Betula pendula*	*Betulaceae*	Eudicots	*Euceraphis betulae*	Piercing		100	10080	-0.05493
Blande et al. (2010)	*Alnus glutinosa*	*Betulaceae*	Eudicots	*Euceraphis betulae*	Piercing		100	10080	-0.00895
Blande et al. (2010)	*Betula pendula*	*Betulaceae*	Eudicots	*Euceraphis betulae*	Piercing		100	10080	0.362061
Blande et al. (2010)	*Betula pendula*	*Betulaceae*	Eudicots	*Euceraphis betulae*	Piercing		100	14400	NA
Blande et al. (2010)	*Betula pendula*	*Betulaceae*	Eudicots	*Euceraphis betulae*	Piercing		100	14400	-0.66969
Blande et al. (2010)	*Betula pendula*	*Betulaceae*	Eudicots	*Euceraphis betulae*	Piercing		100	30240	0.918182
Blande et al. (2010)	*Alnus glutinosa*	*Betulaceae*	Eudicots	*Euceraphis betulae*	Piercing		100	30240	0.911004
Chen et al. (2011)	*Fraxinus nigra*	*Oleaceae*	Eudicots	*Agrilus planipennis*	Chewing		6	14400	0.406802
Chen et al. (2011)	*Fraxinus nigra*	*Oleaceae*	Eudicots	*Agrilus planipennis*	Chewing		6	28800	-1.96724
Chen et al. (2011)	*Fraxinus nigra*	*Oleaceae*	Eudicots	*Agrilus planipennis*	Chewing		6	43200	-1.85553
Chen et al. (2011)	*Fraxinus nigra*	*Oleaceae*	Eudicots	*Agrilus planipennis*	Chewing		6	57600	-1.49272
Chen et al. (2011)	*Fraxinus nigra*	*Oleaceae*	Eudicots	*Agrilus planipennis*	Chewing		6	72000	-1.69089
Chen et al. (2011)	*Fraxinus nigra*	*Oleaceae*	Eudicots	*Agrilus planipennis*	Chewing		6	86400	-1.07812
Colazza et al. (2004)	*Vicia faba*	*Fabaceae*	Eudicots	*Nezara viridula*	Piercing		5	1440	-0.42961
Colazza et al. (2004)	*Phaseolus vulgaris*	*Fabaceae*	Eudicots	*Nezara viridula*	Piercing		5	1440	1.44936
Copolovico et al. (2011)	*Alnus glutinosa*	*Betulaceae*	Eudicots	*Cabera pusaria*	Chewing		4	20	10.16781
Danner et al. (2011)	*Populus trichocarpa*	*Salicaceae*	Eudicots	*Lymantria dispar*	Chewing		50	1020	1.510917

d'Auria et al. (2007)	*Arabidopsis thaliana*	*Brassicaceae*	Eudicots	*Spodoptera exigua*	Chewing		2	240	1.853747
Degenhardt et al. (2010)	*Solanum lycopersicum*	*Solanaceae*	Eudicots	*Manduca sexta*	Chewing		2	1440	1.88388
Degenhardt et al. (2010)	*Solanum lycopersicum*	*Solanaceae*	Eudicots	*Manduca sexta*	Chewing		2	2880	2.425767
Degenhardt et al. (2010)	*Solanum lycopersicum*	*Solanaceae*	Eudicots	*Manduca sexta*	Chewing		2	4320	3.694996
Engelberth et al. (2004)	*Zea mays*	*Poaceae*	Monocots	*Spodoptera exigua*	Chewing	2.5		30	1.123992
Engelberth et al. (2004)	*Zea mays*	*Poaceae*	Monocots	*Spodoptera exigua*	Chewing	2.5		900	4.142461
erb et al. (2015)	*Zea mays*	*Poaceae*	Monocots	*Spodoptera littoralis*	Chewing	10		45	2.750027
Gossner et al. (2014)	*Fagus sylvatica*	*Fagaceae*	Eudicots	*Lymantria dispar*	Chewing		15	240	1.022983
Himanen et al. (2005)	*Fragaria ananassa*	*Rosaceae*	Eudicots	*Galerucella tenella*	Chewing		3	2880	0.212062
Himanen et al. (2005)	*Fragaria ananassa*	*Rosaceae*	Eudicots	*Phytonemus pallidus*	Piercing			2880	3.458469
Himanen et al. (2005)	*Fragaria ananassa*	*Rosaceae*	Eudicots	*Phytonemus pallidus*	Piercing			2880	1.951666
Huang et al. (2009)	*Zea mays*	*Poaceae*	Monocots	*Ostrinia furnacalis*	Chewing		2	2880	3.609358
Kariyat et al. (2012)	*Solanum carolinense*	*Solanaceae*	Eudicots	*Manduca sexta*	Chewing		2	1440	2.31195
Kariyat et al. (2012)	*Solanum carolinense*	*Solanaceae*	Eudicots	*Manduca sexta*	Chewing		2	1440	1.754579
Kessler and Baldwin (2004)	*Nicotiana attenuata*	*Solanaceae*	Eudicots	*Manduca quinquemaculata*	Chewing		1	1440	1.88372
Kessler and Baldwin (2004)	*Nicotiana attenuata*	*Solanaceae*	Eudicots	*Tupiocoris notatus*	Piercing		10	1440	2.252741
Khaling et al. (2016)	*Brassica nigra*	*Brassicaceae*	Eudicots	*Pieris brassicae*	Chewing		30	1440	1.259754
Khaling et al. (2016)	*Brassica nigra*	*Brassicaceae*	Eudicots	*Pieris brassicae*	Chewing		30	4320	1.101138
Li et al. (2015)	*Brassica oleracea*	*Brassicaceae*	Eudicots	*Plutella xylostella*	Chewing		10	1080	2.124471
Li et al. (2015)	*Brassica oleracea*	*Brassicaceae*	Eudicots	*Plutella xylostella*	Chewing		10	1560	-0.77544
Li et al. (2015)	*Brassica oleracea*	*Brassicaceae*	Eudicots	*Plutella xylostella*	Chewing		10	2520	0.618942
McCall et al. (1994)	*Gossypium hirsutum*	*Malvaceae*	Eudicots	*Helicoverpa zea*	Chewing		5	960	1.245637
Michereff et al. (2011)	*Glycine max*	*Fabaceae*	Eudicots	*Euschistus heros*	Piercing		5	4320	0.536142
Michereff et al. (2011)	*Glycine max*	*Fabaceae*	Eudicots	*Euschistus heros*	Piercing		5	4320	-0.27727
Morrison et al. (2016)	*Asparagus officinalis*	*Asparagaceae*	Eudicots	*Agrotis ipsilon*	Chewing			360	0.272064
Ngumbi et al. (2009)	*Gossypium hirsutum*	*Malvaceae*	Eudicots	*Heliothis virescens*	Chewing		30	720	27.04761
Ngumbi et al. (2009)	*Gossypium hirsutum*	*Malvaceae*	Eudicots	*Spodoptera exigua*	Chewing		30	720	28.85439
Penaflor et al. (2011)	*Zea mays*	*Poaceae*	Monocots	*Spodoptera frugiperda*	Chewing	10		1440	4.871376
Piesik et al. (2011a)	*Avena sativa*	*Poaceae*	Monocots	*Oulema cyanella*	Chewing		2	240	4.249524
Piesik et al. (2011a)	*Hordeum vulgare*	*Poaceae*	Monocots	*Oulema cyanella*	Chewing		2	240	15.6101

Piesik et al. (2011a)	*Triticum aestivum*	*Poaceae*	Monocots	*Oulema cyanella*	Chewing	2	240	9.961344
Piesik et al. (2011a)	*Avena sativa*	*Poaceae*	Monocots	*Oulema melanopus*	Chewing	2	240	8.615362
Piesik et al. (2011a)	*Hordeum vulgare*	*Poaceae*	Monocots	*Oulema melanopus*	Chewing	2	240	3.278027
Piesik et al. (2011a)	*Triticum aestivum*	*Poaceae*	Monocots	*Oulema melanopus*	Chewing	2	240	11.77766
Piesik et al. (2015)	*Rumex confertus*	*Polygonaceae*	Eudicots	*Hypera rumicis*	Piercing	10	2880	4.316583
Pinto et al. (2007)	*Brassica oleracea*	*Brassicaceae*	Eudicots	*Plutella xylostella*	Chewing	100	60	2.287284
Pinto-Zevallos et al. (2016)	*Zea mays*	*Poaceae*	Monocots	*Spodoptera frugiperda*	Chewing	10	1440	1.49251
Rodriguez-Saona et al. (2001)	*Gossypium hirsutum*	*Malvaceae*	Eudicots	*Spodoptera exigua*	Chewing	10	1320	3.513392
Rodriguez-Saona et al. (2001)	*Gossypium hirsutum*	*Malvaceae*	Eudicots	*Spodoptera exigua*	Chewing	10	5760	-1.63054
Rodriguez-Saona et al. (2003)	*Gossypium hirsutum*	*Malvaceae*	Eudicots	*Spodoptera exigua*	Chewing	10	1440	2.049924
Rodriguez-Saona et al. (2003)	*Gossypium hirsutum*	*Malvaceae*	Eudicots	*Bemissia tabaci*	Piercing		5760	NA
Rodriguez-Saona et al. (2006)	*Fraxinus mandshurica*	*Oleaceae*	Eudicots	*Agrilus planipennis*	Chewing	10	900	1.278933
Rostas and Turlings (2006)	*Zea mays*	*Poaceae*	Monocots	*Spodoptera littoralis*	Chewing	10	900	6.029459
Rostas et al. (2006)	*Zea mays*	*Poaceae*	Monocots	*Spodoptera littoralis*	Chewing	10	4320	2.87717
Schwartzberg et al. (2011)	*Vicia faba*	*Fabaceae*	Eudicots	*Spodoptera exigua*	Chewing	3	2880	0.645543
Schwartzberg et al. (2011)	*Vicia faba*	*Fabaceae*	Eudicots	*Acyrthosiphon pisum*	Piercing	50	4320	-1.61751
Stanton et al. (2016)	*Nicotiana attenuata*	*Solanaceae*	Eudicots	*Corimelaena extensa*	Chewing		960	-0.04047
Sugimoto et al. (2014)	*Solanum lycopersicum*	*Solanaceae*	Eudicots	*Spodoptera litura*	Chewing	4	1	2.179758
Thaler et al. (2002)	*Solanum lycopersicum*	*Solanaceae*	Eudicots	*Spodoptera exigua*	Chewing	10	600	4.355417
Tooker et al. (2007)	*Triticum aestivum*	*Poaceae*	Monocots	*Mayetiola destructor*	Chewing		1440	0.035756
Tooker et al. (2007)	*Triticum aestivum*	*Poaceae*	Monocots	*Heliothis virescens*	Chewing		7200	1.937277
Verheggen et al. (2013)	*Brassica napa*	*Brassicaceae*	Eudicots	*Heliothis virescens*	Chewing	10	1440	2.119678
Verheggen et al. (2013)	*Brassica napa*	*Brassicaceae*	Eudicots	*Myzus persicae*	Piercing		1440	2.216235
Verheggen et al. (2013)	*Brassica napa*	*Brassicaceae*	Eudicots	*Heliothis virescens*	Chewing	10	4320	2.358801
Verheggen et al. (2013)	*Brassica napa*	*Brassicaceae*	Eudicots	*Myzus persicae*	Piercing		4320	2.422037
Verheggen et al. (2013)	*Brassica napa*	*Brassicaceae*	Eudicots	*Heliothis virescens*	Chewing	10	7200	1.627473
Verheggen et al. (2013)	*Brassica napa*	*Brassicaceae*	Eudicots	*Myzus persicae*	Piercing		7200	2.028181
Wei et al. (2007)	*Vigna unguiculata*	*Fabaceae*	Eudicots	*Liriomyza huidobrensis*	Chewing	125	600	5.104821
Wei et al. (2007)	*Phaseolus lunatus*	*Fabaceae*	Eudicots	*Liriomyza huidobrensis*	Chewing	125	600	1.115438
Wei et al. (2007)	*Phaseolus vulgaris*	*Fabaceae*	Eudicots	*Liriomyza huidobrensis*	Chewing	125	600	-0.46021

Wei et al. (2007)	*Capsicum annuum*	*Solanaceae*	Eudicots	*Liriomyza huidobrensis*	Chewing		125	600	19.54186
Wei et al. (2007)	*Cucurbita sativus*	*Cucurbitaceae*	Eudicots	*Liriomyza huidobrensis*	Chewing		125	600	3.439194
Wei et al. (2007)	*Calendula officinalis*	*Asteraceae*	Eudicots	*Liriomyza huidobrensis*	Chewing		125	600	-0.41312
Williams et al. (2005)	*Zea mays*	*Poaceae*	Monocots	*Nezara viridula*	Piercing		35	1440	-0.38321
Yan et al. (2006)	*Zea mays*	*Poaceae*	Monocots	*Helicoverpa zea*	Chewing	10		60	3.549505
Yan et al. (2006)	*Zea mays*	*Poaceae*	Monocots	*Pseudaletia separata*	Chewing	10		60	6.369488

1645 **Table 3-6: Fungus treatment dataset derived from the meta-analysis using Hedges' g to calculate the treatment effect. Abbreviations: TE, treatment effect. NA: not available.**

Studie	Plant	Family	Eudicots/ Monocots	Pathogen	Minutes after treatment for volatile collection	TE
Piesik et al. (2013)	*Hordeum vulgare*	*Poaceae*	Monocots	*Fusarium spp.*	1440	-0.17215
Piesik et al. (2013)	*Triticum aestivum*	*Poaceae*	Monocots	*Fusarium spp.*	1440	0.247571
Piesik et al. (2015)	*Chrysanthemum morifolium*	*Asteraceae*	Eudicots	*Botrytis cinerea*	4320	42.46706
Piesik et al. (2015)	*Chrysanthemum morifolium*	*Asteraceae*	Eudicots	*Botrytis cinerea*	4320	30.03092
Piesik et al. (2015)	*Chrysanthemum morifolium*	*Asteraceae*	Eudicots	*Botrytis cinerea*	4320	21.28608
Rostas et al. (2006)	*Zea mays*	*Poaceae*	Monocots	*Setosphaeria turcica*	4320	NA
Piesik et al. (2013)	*Hordeum vulgare*	*Poaceae*	Monocots	*Fusarium spp.*	10080	7.550618
Piesik et al. (2013)	*Triticum aestivum*	*Poaceae*	Monocots	*Fusarium spp.*	10080	9.701616
Piesik et al. (2015)	*Chrysanthemum morifolium*	*Asteraceae*	Eudicots	*Botrytis cinerea*	10080	32.69391
Piesik et al. (2015)	*Chrysanthemum morifolium*	*Asteraceae*	Eudicots	*Botrytis cinerea*	10080	22.16647
Piesik et al. (2015)	*Chrysanthemum morifolium*	*Asteraceae*	Eudicots	*Botrytis cinerea*	10080	24.10687
Piesik et al. (2013)	*Hordeum vulgare*	*Poaceae*	Monocots	*Fusarium spp.*	20160	6.926643
Piesik et al. (2013)	*Triticum aestivum*	*Poaceae*	Monocots	*Fusarium spp.*	20160	5.682018
Jiang et al. (2016)	*Populus balsamifera*	*salicaceae*	Eudicots	*Melampsora larici-populina*	1	6.032668
Piesik et al. (2011a)	*Avena sativa*	*Poaceae*	Monocots	*Fusarium culmorum*	240	13.64759
Piesik et al. (2011a)	*Avena sativa*	*Poaceae*	Monocots	*Fusarium graminearum*	240	17.08547
Piesik et al. (2011a)	*Avena sativa*	*Poaceae*	Monocots	*Fusarium avenaceum*	240	8.791366
Piesik et al. (2011a)	*Hordeum vulgare*	*Poaceae*	Monocots	*Fusarium culmorum*	240	10.47191
Piesik et al. (2011a)	*Hordeum vulgare*	*Poaceae*	Monocots	*Fusarium graminearum*	240	9.633426
Piesik et al. (2011a)	*Hordeum vulgare*	*Poaceae*	Monocots	*Fusarium avenaceum*	240	8.760734
Piesik et al. (2011a)	*Triticum aestivum*	*Poaceae*	Monocots	*Fusarium culmorum*	240	11.73921
Piesik et al. (2011a)	*Triticum aestivum*	*Poaceae*	Monocots	*Fusarium graminearum*	240	10.99598

Piesik et al. (2011a)	*Triticum aestivum*	*Poaceae*	Monocots	*Fusarium avenaceum*	240	10.35174
Wenda-Piesik et al. (2010)	*Triticum aestivum*	*Poaceae*	Monocots	*Fusarium spp.*	240	NA
Wenda-Piesik et al. (2010)	*Avena sativa*	*Poaceae*	Monocots	*Fusarium spp.*	240	NA
Wenda-Piesik et al. (2010)	*Hordeum vulgare*	*Poaceae*	Monocots	*Fusarium spp.*	240	NA
Wenda-Piesik et al. (2011)	*Triticum aestivum*	*Poaceae*	Monocots	*Fusarium spp.*	180	2.518718
Wenda-Piesik et al. (2011)	*Triticum aestivum*	*Poaceae*	Monocots	*Fusarium spp.*	180	3.879843
Wenda-Piesik et al. (2011)	*Triticum aestivum*	*Poaceae*	Monocots	*Fusarium spp.*	180	0.304175

Table 3-7: Wounding treatment dataset derived from the meta-analysis using Hedges' g to calculate the treatment effect. Abbreviations: TE, treatment effect.

Study	Plant	Family	Eudicots/ Monocots	Wounding Treatment	Minutes after treatment for volatile collection	TE
Beck et al. (2015)	*Centaurea solstitialis*	*Asteraceae*	Eudicots	needle puncture	1	0.865383
Beck et al. (2015)	*Centaurea solstitialis*	*Asteraceae*	Eudicots	needle puncture	1	-0.30889
Mattiacci et al. (2001)	*Brassica oleracea*	*Brassicaceae*	Eudicots	hole punch	1440	6.608105
Vuorinen et al. (2005)	*Betula pendula*	*Betulaceae*	Eudicots	twig detachment	60	2.774478
Vuorinen et al. (2005)	*Betula pendula*	*Betulaceae*	Eudicots	twig detachment	60	1.64416
Zebelo et al. (2012)	*Solanum lycopersicum*	*Solanaceae*	Eudicots	pattern wheel	60	12.13978
Matsui et al. (2012)	*Arabidopsis thaliana*	*Brassicaceae*	Eudicots	cut	10	0.913958
Engelberth et al. (2004)	*Zea mays*	*Poaceae*	Monocots	cut leaf	30	5.183695
Yan et al. (2006)	*Zea mays*	*Poaceae*	Monocots	leaf scratched	60	4.429087
Allmann et al. (2013)	*Datura wrightii*	*Solanaceae*	Eudicots	pattern wheel	120	4.324755
Allmann et al. (2013)	*Datura wrightii*	*Solanaceae*	Eudicots	pattern wheel	120	3.16885
Allmann et al. (2013)	*Datura wrightii*	*Solanaceae*	Eudicots	pattern wheel	120	2.821418
Engelberth et al. (2004)	*Zea mays*	*Poaceae*	Monocots	cut leaf	900	5.603532
Huang et al. (2009)	*Zea mays*	*Poaceae*	Monocots	leaf perforation	2880	2.77494
Piesik et al. (2015)	*Chrysanthemum morifolium*	*Asteraceae*	Eudicots	needle puncture	4320	11.69302
Piesik et al. (2015)	*Chrysanthemum morifolium*	*Asteraceae*	Eudicots	needle puncture	4320	18.46393
Piesik et al. (2015)	*Chrysanthemum morifolium*	*Asteraceae*	Eudicots	needle puncture	4320	12.64945
Piesik et al. (2015)	*Chrysanthemum morifolium*	*Asteraceae*	Eudicots	needle puncture	10080	10.50555
Piesik et al. (2015)	*Chrysanthemum morifolium*	*Asteraceae*	Eudicots	needle puncture	10080	10.74328
Piesik et al. (2015)	*Chrysanthemum morifolium*	*Asteraceae*	Eudicots	needle puncture	10080	9.52698
Chen et al. (2009)	*Fraxinus pennsylvanica*	*Oleaceae*	Eudicots	stem girdling	20160	0.812508
Chen et al. (2009)	*Fraxinus pennsylvanica*	*Oleaceae*	Eudicots	stem girdling	20160	0.489826
d'Auria et al. (2007)	*Arabidopsis thaliana*	*Brassicaceae*	Eudicots	cut	240	1.077744
Morrison et al. (2016)	*Asparagus officinalis*	*Asparagaceae*	Eudicots	silicon carbide	360	-0.52807
Piesik et al. (2011a)	*Avena sativa*	*Poaceae*	Monocots	poked	240	6.452056

Piesik et al. (2011a)	*Avena sativa*	*Poaceae*	Monocots	scraped	240	5.25153
Piesik et al. (2011a)	*Avena sativa*	*Poaceae*	Monocots	top half	240	2.028352
Piesik et al. (2011a)	*Avena sativa*	*Poaceae*	Monocots	top quarter	240	2.682233
Piesik et al. (2011a)	*Avena sativa*	*Poaceae*	Monocots	bottom quarter	240	3.456508
Piesik et al. (2011a)	*Hordeum vulgare*	*Poaceae*	Monocots	poked	240	5.487468
Piesik et al. (2011a)	*Hordeum vulgare*	*Poaceae*	Monocots	scraped	240	4.739239
Piesik et al. (2011a)	*Hordeum vulgare*	*Poaceae*	Monocots	top half	240	10.33921
Piesik et al. (2011a)	*Hordeum vulgare*	*Poaceae*	Monocots	top quarter	240	5.448652
Piesik et al. (2011a)	*Hordeum vulgare*	*Poaceae*	Monocots	bottom quarter	240	5.487468
Piesik et al. (2011a)	*Triticum aestivum*	*Poaceae*	Monocots	poked	240	5.862609
Piesik et al. (2011a)	*Triticum aestivum*	*Poaceae*	Monocots	scraped	240	4.387757
Piesik et al. (2011a)	*Triticum aestivum*	*Poaceae*	Monocots	top half	240	6.691286
Piesik et al. (2011a)	*Triticum aestivum*	*Poaceae*	Monocots	top quarter	240	4.642754
Piesik et al. (2011a)	*Triticum aestivum*	*Poaceae*	Monocots	bottom quarter	240	4.564232
Wei et al. (2007)	*Vigna unguiculata*	*Fabaceae*	Eudicots	blade cut	600	13.82853
Wei et al. (2007)	*Phaseolus lunatus*	*Fabaceae*	Eudicots	blade cut	600	13.36939
Wei et al. (2007)	*Phaseolus vulgaris*	*Fabaceae*	Eudicots	blade cut	600	1.199027
Wei et al. (2007)	*Solanum lycopersicum*	*Solanaceae*	Eudicots	blade cut	600	1.468894
Wei et al. (2007)	*Capsicum annuum*	*Solanaceae*	Eudicots	blade cut	600	11.33619
Wei et al. (2007)	*Cucurbita sativus*	*Cucurbitaceae*	Eudicots	blade cut	600	1.0277
Wei et al. (2007)	*Calendula officinalis*	*Asteraceae*	Eudicots	blade cut	600	1.071102
Wei et al. (2007)	*Rosa chinensis*	*Rosaceae*	Eudicots	blade cut	600	8.523508
Wei et al. (2007)	*Parthenocissus tricuspidata*	*Vitaceae*	Eudicots	blade cut	600	5.073889

Chapter 4 Priming of wheat with the green leaf volatile Z-3-hexenyl acetate enhances defense against *Fusarium graminearum* but boosts deoxynivalenol production

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Priming of wheat with the green leaf volatile Z-3-hexenyl acetate enhances defense against Fusarium graminearum but boosts deoxynivalenol production

4.1 Abstract

Priming refers to a mechanism whereby plants are sensitized to respond faster and/or more strongly to future pathogen attack. Here, we demonstrate that pre-exposure to the GLV Z-3-hexenyl acetate (Z-3-HAC) primed wheat (*Triticum aestivum* L.) for enhanced defense against subsequent infection with the hemibiotrophic fungus *Fusarium graminearum*.

Bioassays showed that after priming with Z-3-HAC wheat ears accumulated up to 40% less necrotic spikelets. Furthermore, leaves of seedlings showed significantly smaller necrotic lesions compared to nonprimed plants, coinciding with strongly reduced fungal growth *in planta*. Additionally, we found that *F. graminearum* produced more deoxynivalenol (**DON**), a mycotoxin, in the primed treatment. Expression analysis of SA and JA biosynthesis genes and exogenous MeSA and MeJA applications showed that plant defense against *F. graminearum* is sequentially regulated by SA and JA during the early and later stages of infection, respectively. Interestingly, analysis of the effect of Z-3-HAC pre-treatment on SA and JA-responsive gene expression in hormone-treated and pathogen-inoculated seedlings revealed that Z-3-HAC boosts JA-dependent defenses during the necrotrophic infection stage of *F. graminearum* but suppresses SA-regulated defense during its biotrophic phase. Together these findings highlight the importance of temporally separated hormone changes in molding plant health and disease and support a scenario whereby the GLV Z-3-HAC protects wheat against *Fusarium* head blight by priming for enhanced JA-dependent defenses during the necrotrophic stages of infection.

4.2 Introduction

As previously discussed in Chapter 3, BVOCs are known regulators of communication of sedentary plants with their direct environment (Dudareva *et al.*, 2006). Besides attracting pollinators (Pichersky & Gershenzon, 2002), repelling insect herbivores (Birkett *et al.*, 2010) and exerting direct antimicrobial properties (Friedman *et al.*, 2002), BVOCs can act as an alarm signal to warn neighboring plants of an imminent herbivorous or pathogen attack (Heil & Ton, 2008) or serve as an intra-plant signal for the induction of resistance (Karban *et al.*, 2006). Engelberth *et al.* (2004) found that maize seedlings emit the GLVs Z-3-HAL, Z-3-HOL and Z-3-HAC after they had been infested with caterpillars of *Spodoptera exigua*. Neighboring uninfested seedlings which had been exposed to these GLVs, subsequently showed a considerable higher production of the plant defense hormone JA after treatment with caterpillar regurgitant. This form of induced resistance is called priming. Plants in a "primed" state display faster and/or stronger activation of defense pathways when challenged by microbial pathogens, herbivorous insects or abiotic stresses (Conrath, 2009). Exposure to these priming signals does not entail a direct activation of costly defense mechanisms but rather a stronger up-regulation of defense pathways when the plant is actually under attack (van Hulten *et al.*, 2006). Besides resulting in a stronger induction of the JA pathway, priming has also been shown to enhance defense associated with the SA pathway, which plays a critical role in plant defense against biotrophic pathogens (Conrath *et al.*, 2006; Jung *et al.*, 2009).

4.2.1 Fusarium Head Blight: a severe disease with a toxic lifestyle

Fusarium head blight (**FHB**) is an important disease in cereals (Figure 4-1) caused by a complex of *Fusarium* species of which the hemibiotroph *F. graminearum* is one of the most prevalent (Parry *et al.*, 1995; Goswami & Kistler, 2004; Audenaert *et al.*, 2009). Besides yield losses of up to 40%, FHB also confers quality losses because of the production of mycotoxins such as DON (Parry *et al.*, 1995; Bottalico & Perrone, 2002; Vanheule *et al.*, 2014).

Figure 4-1: Fusarium Head Blight (FHB) disease on wheat ears. FHB is characterized by the occurrence of necrotic spikelets in the ear (left) which progresses until the complete ear is affected (right).

Plant defense against the biotrophic and necrotrophic phase has generally been linked to SA and JA related pathways, respectively (Glazebrook, 2005). This was also found in the study by Ding *et al.* (2011). They reported on higher endogenous SA concentration during the first hours of infections, followed by a rise in JA concentration later on. However, plant defense against pathogens is regulated by a whole array of plant hormones between which an intricate crosstalk exists (Pieterse *et al.*, 2012). One of the best studied antagonistic signaling pathways is between SA and JA (Thaler *et al.*, 2002b; Pieterse *et al.*, 2012), which is also preserved in rice, another monocotyledonous crop (De Vleesschauwer *et al.*, 2013; De Vleesschauwer *et al.*, 2014). Because of the presence of this possible antagonistic signaling and the hemibiotrophic lifestyle of *F. graminearum*, it is important to look more closely to the effect of priming on these two defense pathways in wheat.

4.2.2 Toxic secondary metabolites: a fungus' weapon against plant defense

In the continuing arms race between plants and pathogens, pathogenic fungi have evolved several mechanisms to circumvent plant defense. The production of secondary metabolites is a strategy often employed by fungi to facilitate fungal colonization. These fungal metabolites can hijack the plant defense hormone network by inducing antagonistic signaling

pathways which in turn suppresses plant defense against the invading pathogen (Section 2.2.3).

Another group of fungal secondary metabolites are phytotoxins which induce cell death, thus clearing the field for fungal colonization. Fungal toxins can be divided in host selective toxins (**HST**), and non-host selective toxins. HST are generally crucial for the virulence and host specificity of the fungi and are predominately produced by necrotrophic fungi belonging to the order of the Pleosporales (which includes *Alternaria* spp. and *Cochliobolus* spp.) (Pusztahelyi *et al.*, 2015)(Table 4-1). Additionally, plant susceptibility to HST is governed by a toxin target site and thus resistance against HST can be acquired by a change of the target site or detoxification of the HST (Stergiopoulos *et al.*, 2013). Another notorious species of phytotoxin producing fungi are the *Fusarium* spp., which can produce a whole array of toxins (trichothecenes, fumonisins, beauvericine, enniatines) (Bottalico & Perrone, 2002).

Some of these phytotoxins are not only toxic for plants, but are also toxic for animals (Table 4-1), and can have acute, teratogenic, mutagenic, carcinogenic and allergic effects (Desjardins & Hohn, 1997; Bennett & Klich, 2003). This group of secondary metabolites is called mycotoxins. Because of these health concerns, mycotoxin research is of particular interest in studying pathogens infections in crops.

Table 4-1: Non-exhaustive overview of fungal phytotoxins. If no info on animal toxicity for a phytotoxin is available, it is demarked by "–".

Fungus	Phytotoxin	Chemical structure	Mode of action	Animal toxicity	Reference
Fusarium spp.	Thrichothecenes	Sesquiterpene	Inhibition DNA/RNA/ protein synthesis	✓	(McCormick *et al.*, 2011)
	Fumonisins	Polyketide	Inhibition ceramide synthase	✓	(Gilchrist *et al.*, 1995)
	Enniantin/Beauvericin	Cyclic depsipeptide	Disruption of cell membrane	✓	(Jestoi, 2008; Logrieco *et al.*, 2013)
***Alternaria* spp.**	AAL	Polyketide	Inhibition ceramide synthase	✓	(Gilchrist *et al.*, 1995)
	AOH, AME	Dibenzopyrone derivatives		✓	(Ostry, 2008)
	Altertoxins	Perylene derivatives	ROS creation	✓	(Daub *et al.*, 2005)
	Tenuazonic acid	Tetramic acid derivatives	Inhibition of photosystem II	✓	(Chen *et al.*, 2008)
	Tentoxine	Non-ribosomal peptide	Inhibition of chloroplast development	-	(Klotz, 1988)
***Cochliobolus* spp.**	Victorin	Non-ribosomal peptide	Activation of HR response	-	(Tada *et al.*, 2005)
	T-toxin	Polyketide	Disruption of mitochondrial function	-	(Stergiopoulos *et al.*, 2013)
	Prehelminthosporol derivates	Sesquiterpene	Interaction with membrane integrity	-	(Nilsson *et al.*, 1993)
	HC toxin	Non-ribosomal peptide	Inhibition of histone deacetylases	-	(Brosch *et al.*, 1995)
	Ophiobolin	Sesterterpene	Inhibition of calmodulin, interaction with membrane integrity	✓	(Leung *et al.*, 1984)
	HS toxin	Sesquiterpene glycoside	Depolarization of plasma membrane	-	(Schröter *et al.*, 1985)

Abbreviations: AAL: *Alternaria alternata f.sp. lycopersici* toxin; AOH: alternariol; AME: alternariol monomethyl ether; HS-toxin: *Helminthosporium sacchari* toxin; HC toxin: *Helmintosporium carbonum* toxin; T-toxin: *Helminthosporium maydis* (race T) toxin.

4.2.2.1 DON

One of the best studied mycotoxins of the trichothecene group is DON, also called vomitoxin. Its name can be retraced to the emetic effect it has on pigs when DON is ingested (Rotter *et al.*, 1996). At a molecular level, DON inhibits mRNA translation and at high doses DON can induce apoptosis (Pestka, 2010). While the role of mycotoxins in the pathogenesis of fungi is not always known, the role of DON in the infection process of *F. graminearum* is better understood. DON is known to play a role in the switch from biotrophy to necrotrophy of *F. graminearum* (Audenaert *et al.*, 2013)(Figure 4-2).

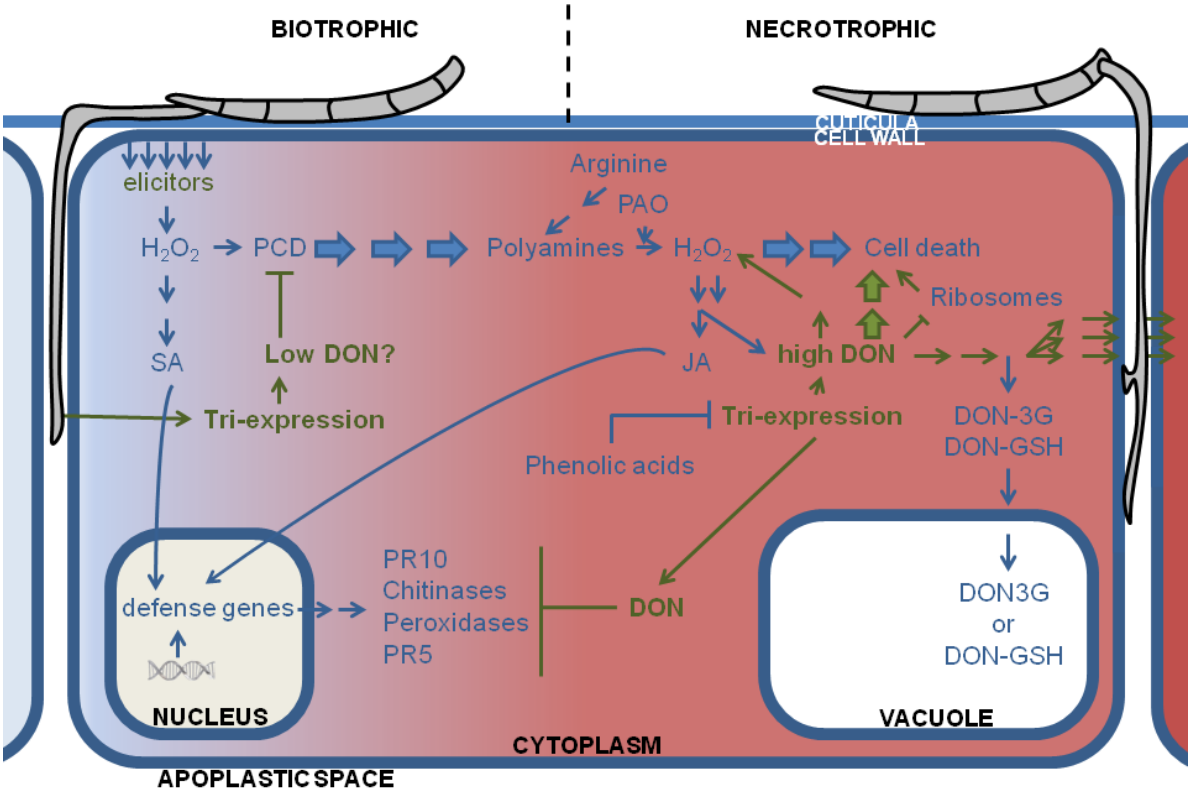


Figure 4-2: Hypothetical model highlighting the role of deoxynivalenol in the infection process of *F. graminearum*. During the biotrophic phase, hyphae grow intercellulary which induces ROS production within the cells. This *triggers F. graminearum* to produce DON which results in a positive feedback loop leading to programmed cell death. DON: deoxynivalenol; DON-3G: DON-glucoside; DON-GSH: DON-gluthatione; JA: jasmonic acid; PAO: polyamine oxidases; PCD: programmed cell death; PR: pathogenesis related; SA: salicylic acid; Tri: trichothecenes (Audenaert *et al.*, 2013).

During the biotrophic phase, spores will germinate and hyphae will grow extra- and intercellulary. To counteract fungal colonization during the biotrophic phase, the host plant will accumulate H_2O_2 to induce programmed cell death. However, H_2O_2 acts as a signal for *F. graminearum* to produce DON which in turn creates a positive feedback loop leading to increased H_2O_2 and DON production, clearing the way for *F. graminearum* to further colonize the host plant (Figure 4-2) (Desmond *et al.*, 2008; Audenaert *et al.*, 2013).

4.2.3 Do GLVs play a role in the *F. graminearum*-wheat interaction?

The lion's share of attention on the use of GLVs in induced resistance has been directed to plant-insect interactions. However, literature regarding priming by GLVs in plant-pathogen interactions remains scarce (Heil, 2014). Few studies have been performed investigating the effect of priming by GLV on plant-fungus interactions (Scala *et al.* (2013a), and references therein). Since the GLVs E-2-HAL, Z-3-HOL, E-2-HOL and Z-3-HAC have also been reported to be emitted by perennial ryegrass after infection with *Fusarium poae* (Panka *et al.*, 2013) or by wheat seedlings after infection with *F. graminearum* (Piesik *et al.*, 2011b), one may speculate that GLVs not only serve as a priming agent against the impending threat of herbivorous insects but rather constitute a general warning and priming mechanism against insects, bacteria and fungi alike. To ascertain whether GLVs are also produced in our model system, we performed a small experiment (in cooperation with ENVOC, Ghent University) in which we measured the GLVs released by wheat ears after *F. graminearum* inoculation (Figure 4-3). One day after inoculation, we did not measure significant differences between control and inoculated ears. However, 1 week after inoculation, we observed an increase in GLVs in the infected ear. Thus, we can conclude that wheat produces GLVs in response to *F. graminearum* infection.

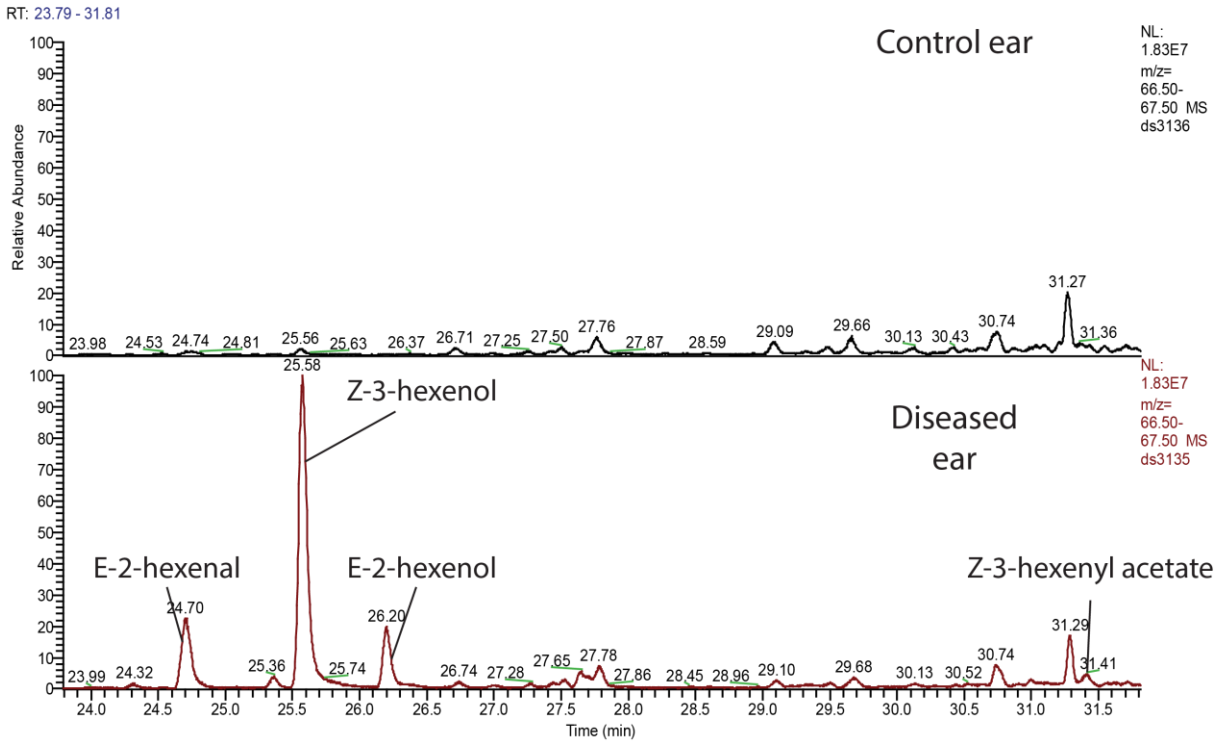


Figure 4-3: Chromatogram of a control ear and an ear which was inoculated with a conidia suspension of *F. graminearum* (1x 10^6 conidia mL^{-1}). BVOCs were captured 1 week after inoculation and analysed using GC-MS.

Priming of wheat with the green leaf volatile Z-3-hexenyl acetate enhances defense against Fusarium graminearum but boosts deoxynivalenol production

In this chapter (Figure 4-4), we will show using bio-assays and RT-qPCR that pre-exposure of wheat to the GLV Z-3-hexenyl acetate primes wheat plants for an enhanced defense against a future infection with *F. graminearum.* Furthermore, our results indicate that pre-treatment with Z-3-HAC leads to a stronger activation of JA related defense while exerting suppressive effects on SA-responsive gene expression. Lastly, we found evidence that enhanced plant defense led to increased DON production by *F. graminearum*.

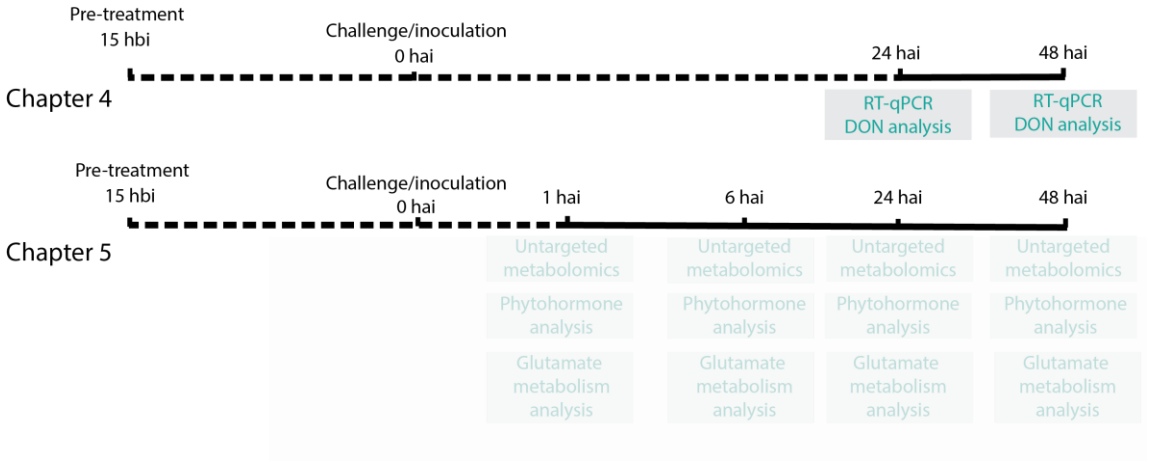


Figure 4-4: Time frame of interest in this chapter and the performed molecular analyses used to investigate wheat-*F. graminearum* interaction. In this chapter we investigate gene expression at 24 and 48 hours after inoculation (hai). Additionally, deoxynivalenol (DON) content was analyzed at these time points. In the following chapter, we will look more closely at earlier time points and additional techniques will be used. Abbreviations: hai, hours after inoculation; hbi, hours before inoculation; DON, deoxynivalenol

4.3 Materials and Methods

4.3.1 *F. graminearum* and conidia spore suspension

A GFP transformant of *F. graminearum* strain 8/1 and a *Tri5* knockout mutant (Jansen *et al.*, 2005) (kindly provided by dr. Karl Heinz-Kogel) were grown on potato dextrose agar for seven to ten days at 20°C under a regime of 12h dark and 12h combined UVC and UVA light (2x TUV 8W T5 and 1x TL 8W BLB, Philips, the Netherlands). Macronidia were harvested by adding a solution of 0.01% Tween80 to the PDA plates and rubbing the mycelium with a drigalski spatula. Subsequently, the suspension was diluted to a final concentration of 5×10^5 conidia ml^{-1}.

4.3.2 Plant material

Six seeds of wheat (*Triticum aestivum* L.) var. Sahara were germinated and grown in pots (8.5 cm diameter x 6.5 cm height) in a growth chamber (18°C, 16 h-8 h, light-dark regime) for

two weeks. When the seedlings reached wheat GS 12 (Lancashire *et al.*, 1991), plants were selected for the leaf sheath bio-assay.

4.3.3 BVOC exposure system

In this and other chapters the same exposure system was used.

In order to treat plants or plant parts with a volatile compound, we built an exposure system based on a system described in Joo *et al.* (2010). A modified push-pull system (Tholl *et al.*, 2006) was built in which we supplied an enclosed plant, or a plant part with purified air. Excess air could exit through a vent (Figure 4-5).

To examine the effect of a volatile compound on plant physiology, a constant flow of purified air is mandatory. Furthermore, the materials of which the several components of the system consist need to be inert. This implies that the materials do not interact with the BVOC or that the BVOC cannot be ab- or adsorbed by the material. Additionally, it is preferable that the materials do not release volatile compounds which may exert an effect on the plants. Glass and other plastics such as Teflon, nalophan and Tedlar are recommended in plant volatile headspace collection systems (Tholl *et al.*, 2006). We used 4 liter bags made of nalophan (NA 300, Foodpack, Harderwijk, the Netherlands) in our system, a material commonly used in olfactometry tests and gas sampling and which is recommended by European and American standards for olfactometry panel assays (CEN, 2003; ASTM, 2004). Tubing and connections were made out of perfluoroalkoxy alkane (PFA) or stainless steel (Swagelok, Solon, OH, USA). Air is drawn through the system by a membrane vacuum pump (maximum flow: 30 L min^{-1}; N035AN.18, KNF Neuberger GmbH, Freiburg i. Br., Germany). To purify the air of pollutants and ozone, which can degrade BVOCs, air was pulled over two stainless steel canisters (50 cm x 8.8 cm o.d.) containing active carbon (Airpel 10, Desotec, Roeselare, Belgium) and an ozone filter (ETO342FC002A, Ansyco, Karlsruhe, Germany). To prevent possible recirculation of air which was vented out of the nalophan cuvettes, the inlet tube of the charcoal filters was placed outside the growth chamber. Additionally, a dust filter was installed after the active carbon canisters and the ozone filter. To achieve a constant air flow through the cuvettes we used mass flow controllers (MFCs) (GF40, Brooks Instruments, Hatfield, CA, USA). A programmable logic controller (PLC) was designed and developed with help of Erik Moerman (Laboratory of Plant Ecology, Ghent University) to control the MFCs. Flow throughout the cuvettes was maintained at 600 mL min^{-1}.

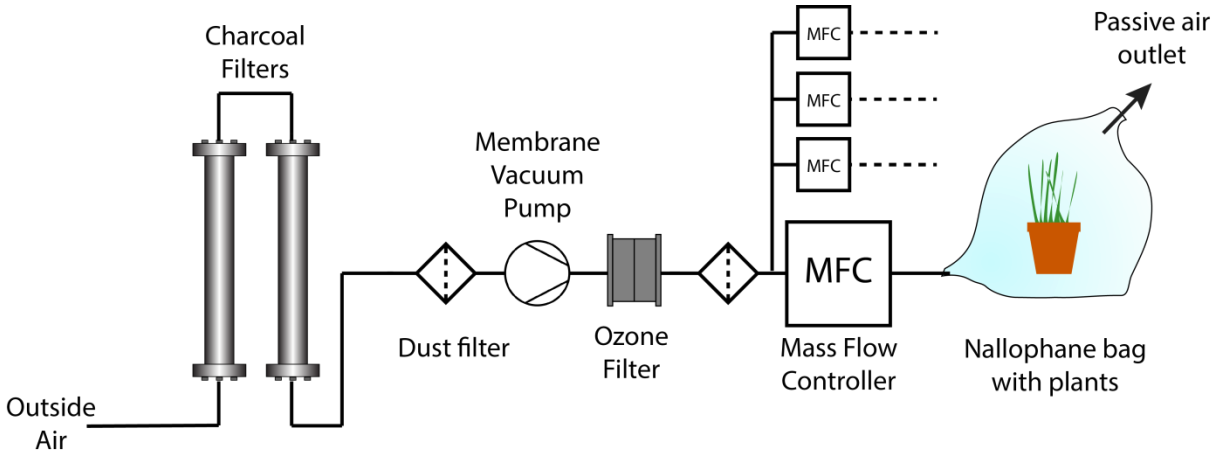


Figure 4-5: Diagram depicting the cuvette system used throughout this doctoral thesis. To supply the cuvettes with sufficient air, we used a slightly modified design by Joo *et al.* (2010). A membrane pump (N035AN.18, KNF Neuberger GmbH, Freiburg i. Br., Germany), which was installed after a dust filter (2 µm pore size Zefluor PTFE Membrane Filter, Pall, MI, USA), provided a continuous air stream of 600 ml min^{-1} (GF40, Brooks Instruments, Hatfield, CA, USA). In order to purify the incoming air of pollutants and ozone, air passed through two active carbon filters (Airpel 10, Desotec, Roeselare, Belgium) and an ozone filter (ETO342FC002A, Ansyco, Karlsruhe, Germany), respectively. Tubing and connections were made out of stainless steel or perfluoroalkoxy alkane (Swagelok, Solon, OH, USA).

4.3.4 Experimental design

We designate primed plants as wheat plants which have been pre-exposed to Z-3-HAC. Unless stated otherwise, four different treatments were used. (1) A control treatment, (2) a priming treatment in which wheat plants were primed with Z-3-HAC (Sigma-Aldrich, ≥98% purity), (3) a treatment in which primed wheat plants were subsequently challenged with a conidia suspension of *F. graminearum*, MeSA (Sigma-Aldrich) or MeJA (Sigma-Aldrich), (4) a treatment in which nonprimed plants were challenged with a conidia suspension of *F. graminearum.* These treatments were additionally expanded to include treatments in which we pre-exposed seedlings to MeSA or MeJA followed by a subsequent challenge with *F. graminearum* and finally treatments in which Z-3-HAC treated seedlings were subsequently challenged with MeSA and MeJA. In the bar charts in the result section, specific colors will be designated to specific combination of treatments (Table 4-2).

Table 4-2: Color scheme for the different treatments used in this study. Rows represent whether seedlings have been pre-exposed to Z-3-HAC, MeSA, MeJA or not. Columns represent the different challenges after pre-exposure

Treatment\Challenge	Mock	Fusarium graminearum	MeSA	MeJA
Mock				
Z-3-HAC			Striped yellow	Striped blue
MeSA		Striped yellow		
MeJA		Striped blue		

To expose wheat to Z-3-HAC, a dynamic push-pull cuvette system was used as previously described (Figure 4-5). Wheat plants were placed in one of four nalophan cuvettes, which were assigned to one of the abovementioned treatments (Figure 4-6). We applied 70 µl of Z-3-HAC (≥98% purity, Sigma-Aldrich) on a piece of filter paper inside the two cuvettes which were assigned to the priming treatment, (2) + (3) (5 PM). Using this set-up, plants were exposed for a short amount of time to a concentration of maximum 0.11 mM Z-3-HAC (see section 4.8) which dropped rapidly to previously reported values in wheat after infection with *F. graminearum* (Piesik *et al.*, 2011a; Piesik *et al.*, 2011b; Wenda-Piesik, 2011).To eliminate a direct effect of Z-3-HAC on *F. graminearum*, the filter paper was removed the following day (8 AM) prior to inoculation. Additionally, the cuvettes were allowed to flush in order to eliminate trace amounts of Z-3-HAC. This ensures that Z-3-HAC does not directly influence fungal growth or wheat growth (See section 4.7). Subsequently, the plants were challenged with a suspension of *F. graminearum*, or to 70 µL of MeSA or MeJA, which was pipetted on a piece of filter paper, according to the respective treatments, (3) + (4). Analogous to the Z-3-HAC application, MeSA and MeJA reached maximally concentrations of 0.14 and 0.08 mM, respectively. At different time points, disease severity was scored and samples were taken for further analysis (Figure 4-6).

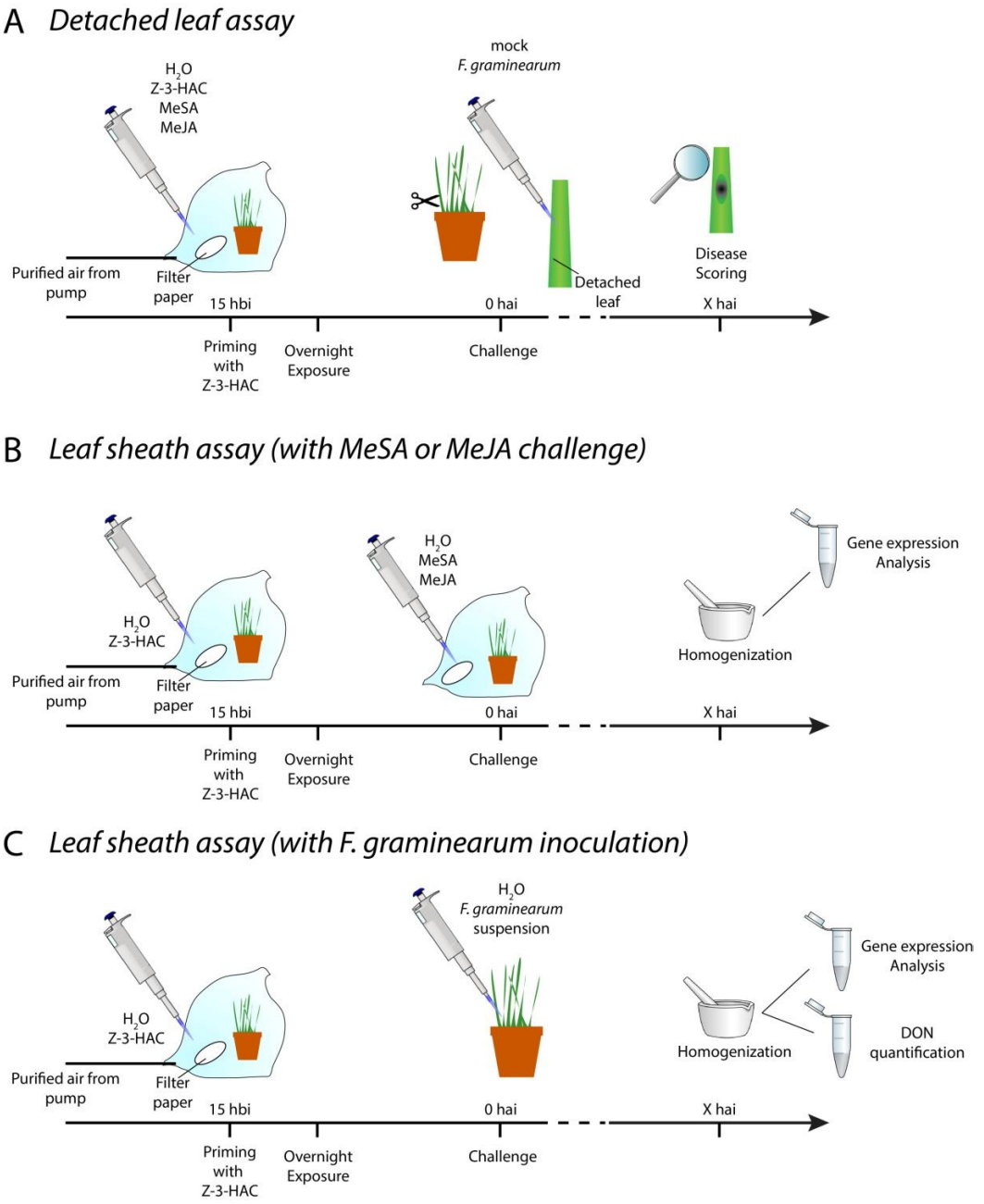


Figure 4-6: Diagram depicting the general design of the different experiments. A. Detached leaf assay: seedlings were placed in nalophan cuvettes and a mock treatment, Z-3-HAC, methyl salicylate (MeSA) or methyl jasmonate (MeJA) was applied on a piece of filter paper according to the experiment. A continuous air flow (600 ml min^{-1}) was supplied to the cuvettes to prevent an increase of the relative humidity. Seedlings were kept inside the cuvettes overnight and the day after, leaves were cut from the plant and placed in Petri dishes. Leaves were subsequently inoculated with a mock treatment or a conidia suspension of *F. graminearum*. The following days, lesion length was measured. **B.** Leaf sheath assay: seedlings were placed in nalophan cuvettes and a mock treatment or Z-3-HAC was applied on a piece of filter paper according to the experiment. A continuous air flow (600 ml min^{-1}) was supplied to the cuvettes to prevent an increase of the relative humidity. Seedlings were kept inside the bags overnight and the day after, the cuvettes were flushed and seedlings were challenged by aerial treatment with MeSA or MeJA, which was pipetted on a piece of filter paper. The following days, leaf sheaths were sampled and prepared for gene expression analysis. **C**. Leaf sheath assay with a *F. graminearum* inoculation. seedlings were placed in bags and a mock treatment or Z-3-HAC was applied on a piece of filter paper according to the experiment. A continuous air flow (600 ml min^{-1}) was supplied to the cuvette to prevent an increase of the relative humidity. Seedlings were kept inside the cuvettes overnight and the day after, seedlings were taken from the cuvettes. Leaf sheaths were subsequently challenged with a mock treatment or a conidia suspension of *F. graminearum.* The following days, leaf sheaths were sampled and prepared for gene expression analysis and deoxynivalenol quantification. Abbreviations: Z-3-HAC, Z-3-hexenyl acetate; hai, hours after inoculation; hbi, hours before inoculation, MeSA, methyl salicylate; MeJA, methyl jasmonate; DON, deoxynivalenol.

4.3.5 Ear infection experiment

To evaluate the effect of a pre-exposure to the GLV Z-3-HAC on infection of wheat ears by *F. graminearum*, we performed an infection assay. We placed a total of nine wheat ears var. Sahara for each treatment in cuvettes. We applied 70 µl of Z-3-HAC (Sigma-Aldrich) on a piece of filter paper inside the cuvette which was assigned to the priming treatment and removed it the following day. Subsequently, we point inoculated three spikelets of each ear with 20 µl of a suspension of 5 x 10^5 conidia ml^{-1} per spikelet. Every two days, we evaluated the inoculated spikelets for signs of necrosis.

4.3.6 Detached leaf assay

To investigate whether pre-exposure of wheat seedlings to Z-3-HAC, MeSA or MeJA (Sigma-Aldrich) leads to enhanced defense against a subsequent infection by *F. graminearum*, we performed a detached leaf assay experiment following Imathiu *et al.* (2009)(Figure 4-6A). In short, after seedlings were pre-exposed to 70 µl Z-3-HAC, MeSA or MeJA inside the cuvettes as previously described, 4 cm leaf segments (n=15) were cut from the tip of the leaves of seedlings at GS 12. These leaves were placed on their abaxial surface in Petri dishes containing 0.5% bacteriological water agar amended with 40 mg l^{-1} benzimidazole, which delays leaf senescence (Mishra & Pradhan, 1973). The center of the leaf segment was wounded using a sterile inoculation needle after which a droplet of conidia suspension of 5 x 10^5 conidia ml^{-1} was placed on the wound. Lesion length was measured the following days using Cell^F (Olympus).

4.3.7 Leaf sheath bio-assay

To evaluate the expression of defense genes of wheat after *F. graminearum* infection, a leaf sheath bio-assay, based on Koga *et al.* (2004), was used (Figure 4-6). This allows for a minimal wound response on gene expression. In short, after overnight exposure to Z-3-HAC in the cuvettes, the leaf sheath of the first leaf was carefully peeled off, while still remaining attached to the plant. Afterwards the curved cavity was filled with the conidia suspension of 5 x 10^5 conidia ml^{-1} or water, according to the respective treatments. The inoculated seedlings were subsequently placed back inside the cuvette. After an incubation period of 24 and 48 hours, leaf sheaths from the different treatments were excised and flash frozen in liquid nitrogen and stored at -80°C for later RNA extraction.

To establish a time point for fungal penetration of the plant cell wall, microscopic images were taken at different time points from inoculated (5 x 10^5 conidia ml^{-1}) leaf sheaths after they have been submerged in 0.05% aniline blue dye prepared in lactic acid for 30 min (Audenaert *et al.*, 2010).

4.3.8 RNA extraction, RT-qPCR

RNA from the leaf sheaths was extracted using TRI reagent (Sigma-Aldrich, Saint-Louis, MO, USA) according to the manufacturer's specifications and quantified with a spectrophotometer (ND1000, Nanodrop, Wilmington, DE, USA). For each sample, four leaf sheaths were pooled, to a total of three to four biological replicates. First-strand cDNA was synthesized from 500 ng total RNA, using the Superscript III First-Strand Synthesis Supermix kit (Life technologies, Carlsbad, CA, USA). The presence of genomic DNA was checked using gel electrophoresis. The primers used for RT-qPCR analysis are listed in Table 4-3. RT-qPCR analysis was performed using a CFX96 system (Bio-Rad, Hercules, CA, USA). The thermal profile consisted of an initial denaturation step for 3 min at 95°C, followed by 40 cycles of 95°C for 30 s and 60°C for 60 s. Finally, melting curve analysis was performed using a temperature profile of 95°C for 10 s, cooling to 65°C for 5 s, subsequently heating to 95°C at a rate of 0.5°C per 10 s.

Table 4-3: Primers used for RT-qPCR

Gene	Forward (5'-3')	Reverse (5'-3')	Reference
FGSG_01244	CTAGCAACTTTCCGCGATGC	CCGTCCACAAGTCGACAGAA	This Chapter
LOX1	GGCACGCCATCGAGCAGTACG	TACTGCCCGAAGTTGACCGCC	Feng *et al.* (2010)
LOX2	AACAAGTTCGCCGTCACCTT	TTGTCGAGGGTGATGGTCTT	Beccari *et al.* (2011)
PR1	CGTCTTCATCACCTGCAACTA	CAAACATAAACACACGCACGTA	Gao *et al.* (2013)
PR2	CCGCACAAGACACCTCAAGATA	CGATGCCCTTGGTTTGGTAGA	Gao *et al.* (2013)
PR4	ACACCGTCTTCACCAAGATCGACA	AGCATGGATCAGTCTCAGTGCTCA	Qi *et al.* (2012)
PR5	ACAGCTACGCCAAGGACGAC	CGCGTCCTAATCTAAGGGCAG	Gao *et al.* (2013)
Ta54227	CAAATACGCCATCAGGGAGAACATC	CGCTGCCGAAACCACGAGAC	Paolacci *et al.* (2009)
Ta35284	AGCAATTCGCACAATTATTACAAG	CTCACAGAAGACCTGGAAGC	Paolacci *et al.* (2009)
ICS	AGAAATGAGGACGACGAGTTTGAC	CCAAGTAGTGCTGATCTAATCCCAA	Ding *et al.* (2011)
PAL	TTGATGAAGCCGAAGCAGGACC	ATGGGGGTGCCTTGGAAGTTGC	Ding *et al.* (2011)
PEROX(PRX113)	GAGATTCCACAGATGCAAACGAG	GGAGGCCCTTGTTTCTGAATG	Desmond *et al.* (2005)
NADPHOX	ATGCTCCAGTCCCTCAACCAT	TTCTCCTTGTGGAACTCGAATTT	Ding *et al.* (2011)
CAD1	AGATACCGCTTCGTCATCG	GAATCGCACGCACCAACC	Bi *et al.* (2011)
CCR3	CTGTCGGCTAGTTAATTCTATG	ATATGATCGCCAACCAACC	Bi *et al.* (2011)

Active fungal biomass was quantified using pre-mRNA slicing factor of *F. graminearum* (*FGSG_01244*) (Becher *et al.*, 2011). Normalization of wheat defense genes was carried out using Cell division control protein (*Ta54227*) and Protein transport protein Sec23A (*Ta35284*) as reference genes (Paolacci *et al.*, 2009). All calculations and analysis of the quality of the reference genes were performed using qBase+ software (Biogazelle NV, Zwijnaarde, Belgium).

4.3.9 DON quantification

4.3.9.1 Sample preparation

To investigate whether pre-exposure of wheat seedlings to Z-3-HAC will impact DON production by *F. graminearum* or phytohormone production by plants, DON was measured using U-HPLC-MS based on a method described by Van Meulebroek *et al.* (2012). In short, 200 mg of 6 to 8 pooled leaf sheaths were crushed using liquid nitrogen. Afterwards, 1 ml of cold modified Bieleski extraction buffer (-20 °C) consisting of methanol, ultrapure water and formic acid (75:20:5, v/v/v) was added. Additionally, the suspension was amended with a deuterium labeled internal standard of 100 pg μl^{-1} d_6-abscicic acid (OlChemIm, Olomouc, Czech Republic). Subsequently, the samples were vortexed and placed at -20 °C for 12h of cold extraction. The samples were centrifuged and 500 µl of the supernatant was transferred to a 30 kDa Amicon® Ultra centrifugal filter unit (Merck, Millipore Corporation, Massachusetts, USA). The purified extract was subsequently reduced under vacuum at 35°C to a fourth of the original volume (Gyrovap, Howe, Banbury, UK). Finally, the extract was transferred to an HPLC vial and 10 µl was injected directly on column.

4.3.9.2 U-HPLC-MS

The U-HPLC-MS system consisted of an Accela U-HPLC pumping system (Thermo Fisher Scientific, San Jose, USA), coupled to an Exactive™ Orbitrap mass spectrometer (Thermo Fisher Scientific, San Jose, USA) and equipped with a heated electrospray ionization source (HESI), operating in both the positive and negative mode (switching polarity mode). Chromatographic separation of the compounds was achieved with a gradient elution program, using a reversed phase Nucleodur Gravity C18 column (1.8 µm, 50 mm × 2.1 mm ID) (Macherey-Nagel, Düren, Germany). The column oven temperature was set at 30 °C. The mobile phase consisted of a binary solvent system: 0.1% formic acid in ultrapure water (solvent A) and methanol (solvent B) at a constant flow rate of 300 µl min^{-1}. A linear gradient profile with the following proportions (v/v) of solvent A was applied: 0–1 min at 98%, 1–2.50 min from 98 to 60%, 2.50–4 min from 60 to 50%, 4–5 min from 50 to 20%, 5–7 min at 20%,

7–7.10 min from 20 to 0%, 7.10–8 min at 0%, 8–8.01 min from 0 to 98%, followed by 2 min of re-equilibration. The instrumental parameters for HESI can be found in Van Meulebroek *et al.* (2012). DON was identified based on both the retention time relative to the internal standard and the accurate mass (m/z: 197.1337, positive ionization mode). After identification, concentrations were calculated by fitting the area ratios into a seven-point calibration curve, set up in a leaf sheath matrix. DON was kindly provided by dr. Marc Lemmens.

Table 4-4: Accurate masses, ionization modes and retention times used for the identification of DON and the internal standard.

Analyte	Ionization modus	Accurate mass *(m/z)*	Retention Time (min)
DON	+	297.1337	3.38
D6-ABA (Int. Std.)	-	269.1665	5.25

4.3.10 Data analysis

Gene expression data were checked for normality using the Shapiro Wilk test, equality of variances was checked using Levene's test. Statistical comparisons between different treatments were calculated using proc mixed (SAS 9.0). Statistical differences between the primed and nonprimed treatments in the spikelet infection experiment were calculated using the χ^2 -test (SPSS 20; IBM).

4.4 Results

4.4.1 Effect of Z-3-HAC pre-treatment on the severity of infection by *F. graminearum*

To assess whether pre-exposure to Z-3-HAC results in increased resistance against infection with *F. graminearum*, we point-inoculated 27 spikelets of nine wheat ears, the target tissue of *F. graminearum* with 20 µl of a conidia suspension for the pre-exposed and control treatment. Four days after infection, we observed the first necrotic lesions (Figure 4-7A). The pre-exposed treatment exhibited a significantly lower infection rate than the control treatment until 6 days post inoculation. All inoculated spikelets showed necrosis 8 days after inoculation for both treatments.

Previously, Purahong *et al.* (2012) reported on the high correlation between FHB resistance levels of wheat ears in field trials and those of detached leaves in a Petri-dish bio-assay. Given the experimental tractability of the latter assays, we next tested the ability of Z-3-HAC

to reduce FHB development and severity in a series of detached leaf experiments. At 24 hai, we did not find significant differences in lesion length between pre-exposed and control seedlings. However, at 48 hai and 72 hai, lesion length was significantly larger (+20%, $P<0.05$ and +72%, $P<0.01$, respectively) in control seedlings, compared to pre-exposed seedlings (Figure 4-7B). Additionally, lesions of pre-exposed seedlings showed an easily distinguishable front of dark necrotic cells, while control seedlings showed more water-soaked lesions (Figure 4-7C).

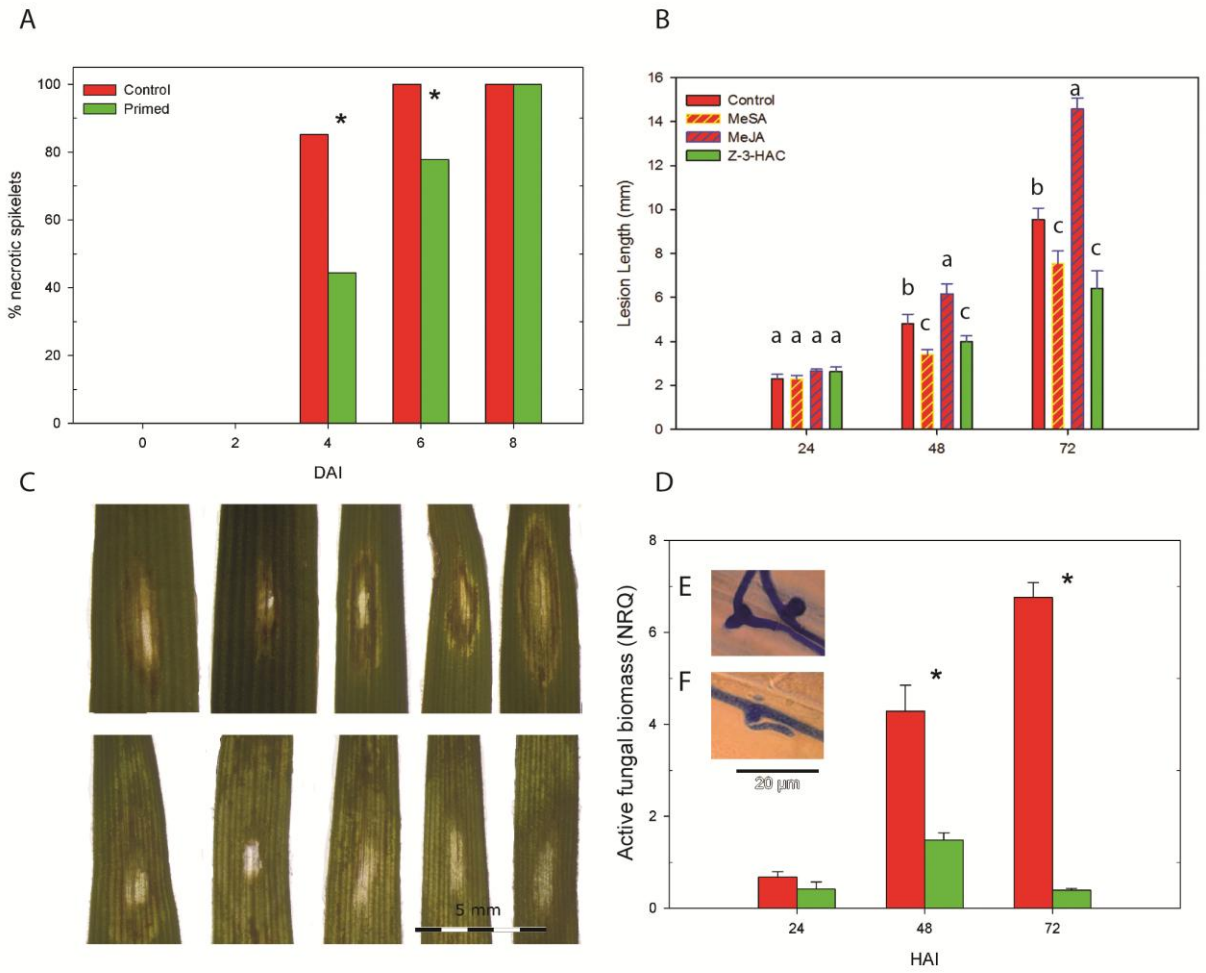


Figure 4-7: Pre-exposure with Z-3-HAC leads to lower fungal biomass and smaller necrotic lesions in wheat seedlings. A, Percentage of spikelets (n = 27) showing necrotic lesions after pre-treatment with Z-3-HAC at 0, 2 4, 6, and 8 days after inoculation (DAI). Significant differences between treatments are depicted with asterisks. Significance was determined using the χ^2 test with a significance level of 0.05. B, Leaves of seedlings pre-exposed to Z-3-HAC or MeSA show smaller necrotic lesions compared with nonprimed control seedlings, while pre-exposure to MeJA exacerbates lesion length. Leaves were cut from the seedlings and subsequently wounded, after which a droplet of a conidia suspension of *F. graminearum* (5 x 10^4 conidia mL^{-1}) was applied on the wound. Lesion length was monitored at 24, 48, and 72 hai. Bars represent means of 15 biological replicates. Bars depicted with different letters per time point indicate significant differences between the treatments ($P < 0.05$). Error bars represent SE. Statistical differences were calculated using One-Way ANOVA with a post-hoc Tukey test. C, Photographs depicting representative necrosis symptoms at 72 hai. The top row shows leaves that have been primed with Z-3-HAC, while the bottom row shows leaves that have not been primed. D, Normalized quantitative relative values (NRQ) of fungal biomass. Leaf sheaths were exposed overnight to Z-3-HAC. The next day, a conidia suspension of *F. graminearum* (5 x 10^4 conidia mL^{-1}) was applied in the leaf sheaths. Biomass was determined using premRNA slicing factor of *F. graminearum* (*FGSG_01244*) as a reference gene and expressed relative to the plant reference genes cell division control protein (*Ta54227*) and protein transport protein Sec23A (*Ta35284*). Bars represent means of two biological replicates of four pooled leaf sheaths each. E and F, Microscopic images illustrate the formation of infection structures at 24 hai (E) followed by invasion of the plant cell^F

To investigate the effect of seedling pre-exposure to Z-3-HAC on fungal growth, we inoculated leaf sheaths with a conidia suspension using the leaf sheath bio-assay. At each time point we consistently found lower active fungal biomass in the pre-exposed seedlings as compared to the control treatment (24 hai: -39%, P=0.25; 48 hai: -65%, P<0.05; 72 hai: -94%, P<0.05) (Figure 4-7D).

We inoculated leaf sheaths of wheat seedlings with a conidia suspension in order to establish the time point at which the fungal hyphae form infection structures and invade the plant cell wall. After an incubation period of 24 h, we found a large formation of appresoria-like structures and foot structures (Jansen *et al.*, 2005; Boenisch & Schaefer, 2011) (Figure 4-7 E,F) and decided to take this time point as a starting point for further experiments.

4.4.2 Expression of JA and SA biosynthesis genes after *F. graminearum* inoculation

Because of the hemibiotrophic lifestyle of *F. graminearum*, we verified whether a sequential upregulation of the biosynthesis genes for the SA and JA pathway was present. We selected phenylalanine ammonia lyase (***PAL***) and isochorismate synthase (***ICS***) as marker genes for the biosynthesis of SA (Ding *et al.*, 2011) and lipoxygenase 1 and 2 (*LOX1* and *LOX2*) as marker genes for the biosynthesis of JA (Feng *et al.*, 2010). Using the leaf sheath bio-assay, we inoculated seedlings with a conidia suspension of *F. graminearum.* Expression analysis revealed at 24 hai a significant upregulation of *PAL* ($P < 0.05$) and a significant downregulation of *LOX2* ($P < 0.05$), while expression of *ICS* and *LOX1* was not significantly different from the control treatment (Figure 4-8). However, at 48 hai, we saw a significant upregulation of *LOX1* and *LOX2* ($P < 0.05$), while expression of *PAL* was not different from the control and *ICS* was even significantly downregulated ($P<0.05$) (Figure 4-8).

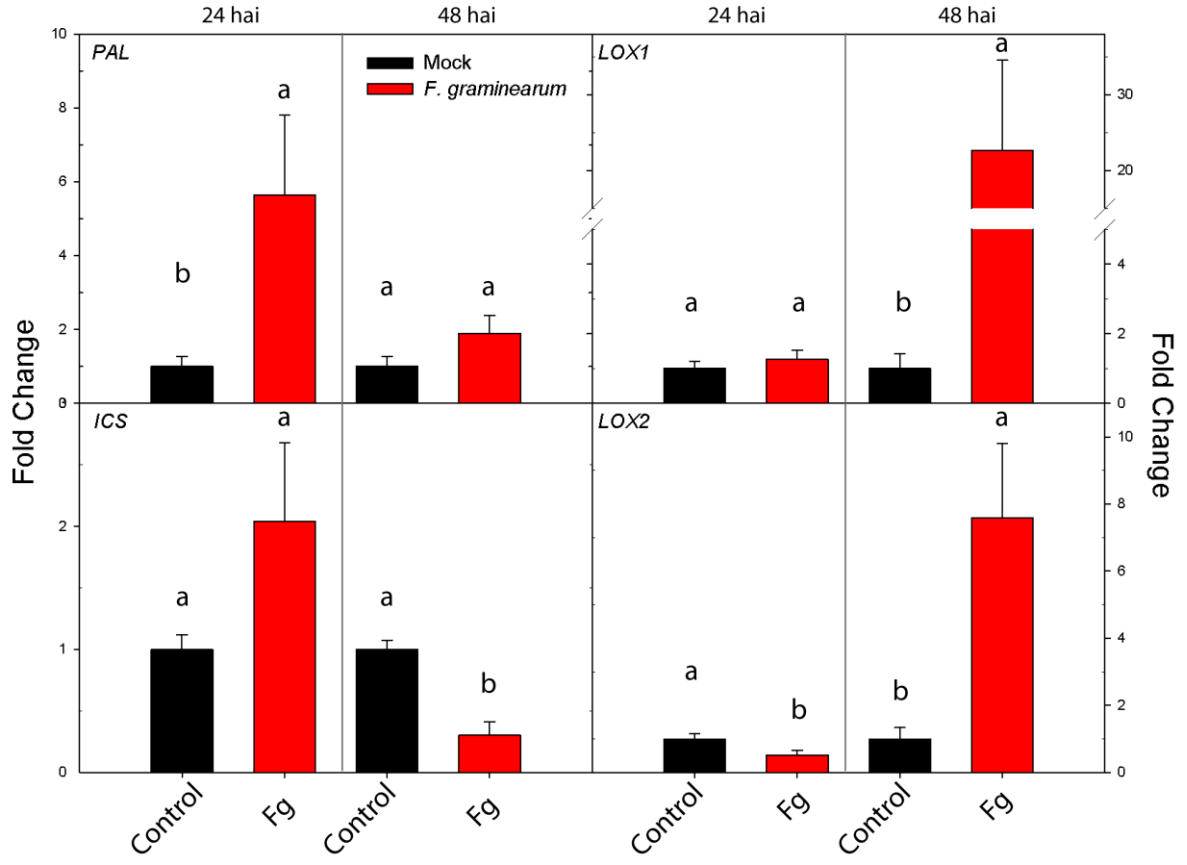


Figure 4-8: Expression profiles of *PAL*, *ICS*, *LOX1*, and *LOX2* at 24 and 48 h after inoculation with a conidia suspension of *F. graminearum*. Leaf sheaths were inoculated with a conidia suspension (5 x 10^4 conidia ml^{-1}). At 24 and 48 hai, leaf sheaths were excised and flash frozen in liquid nitrogen for later RNA extraction and qRT-PCR. Data represent means of four biological replicates, each consisting of four pooled leaf sheaths. Error bars represent SE. Different letters above the bars indicate significant differences between the treatments per time point. Statistical differences were calculated by performing a generalized linear model (GLM) procedure (α = 0.05).

4.4.3 Effect of exogenous MeSA and MeJA on disease development

As defense against *F. graminearum* has been attributed to both SA- and JA-related defense pathways and following our previous results, we assessed whether pre-exposure to MeSA or MeJA, contributed to smaller lesions in leaves of infected wheat seedlings. Remarkably, while seedlings which had been pre-exposed to MeSA exhibited significantly smaller lesions at 48 hai (-29%, P<0.05) and 72 hai (-21%, P<0.05) compared to the control seedlings (Figure 4-7B), pre-exposure to MeJA led to significantly longer lesions at 48 hai (+29%, P<0.05) and 72 hai (+53%, P<0.05), showing enhanced susceptibility (Figure 4-7B). However, because our previous observations showed an induction of JA biosynthesis genes between 24 and 48 hai, we verified whether treating the seedlings with MeJA at 24 hai would lead to enhanced defense. At 24 hai and 48 hai there were no significant differences between the treatments (Figure 4-9). Nonetheless, at 72 hai, the treatment with MeJA led to lower lesion length. Additionally, lesion length for the seedlings which have been pre-

exposed to Z-3-HAC was even lower (-57%, $P < 0.05$) than the non-pre-exposed seedlings treated with MeJA (-20%, $P < 0.05$) (Figure 4-9).

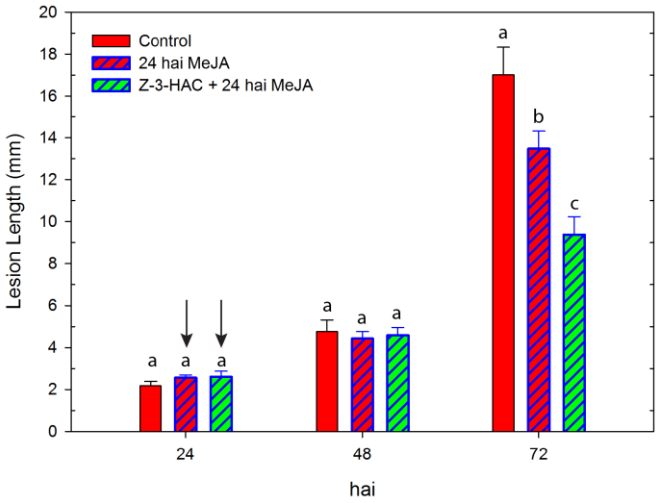


Figure 4-9: Leaves of seedlings pre-exposed to Z-3-HAC and treated with MeJA at 24 hai show smaller lesions compared with the control treatment. Leaves were cut from the seedlings and subsequently wounded, after which a droplet of a conidia suspension of *F. graminearum* (5 x 10^4 conidia mL^{-1}) was applied on the wound. Lesion length was monitored at 24, 48, and 72 hai. Arrows indicate the time points at which the seedlings were treated with 10 µL of MeJA applied on a filter paper. Bars represent means of 10 to 15 biological replicates. Bars depicted with different letters per time point indicate significant differences between the treatments (α=0.05). Error bars represent SE. Abbreviations: hai, hours after inoculation; MeJA, methyl jasmonate.

In conjunction with our gene expression results, these observations support an important role of both SA and JA in plant defense against *F. graminearum* with SA mainly contributing to resistance during the pathogen's early biotrophic growth and JA conditioning plant immunity during later stages of infection.

4.4.4 Gene expression of pre-exposed seedlings after treatment with MeSA or MeJA

To elucidate whether the pre-exposure of Z-3-HAC leads to a direct activation of plant innate immunity or rather primes for an enhanced defense response following pathogen attack, we first investigated the impact of Z-3-HAC pre-exposure on MeSA- and MeJA-inducible gene expression.

We selected *PAL* and *ICS* as marker genes for the salicylate pathway (Ding *et al.*, 2011) and *LOX1* and *LOX2* for the jasmonate pathway (Feng *et al.*, 2010). Additionally, we selected different plant defense genes encoding pathogenesis related proteins which are known to play a role in the defense against infection with *F. graminearum*. Namely, the pathogenesis related proteins: *PR1* (Makandar *et al.*, 2012), *PR2,* β-1,3-glucanase (Gao *et al.*, 2013), *PR4*, shown to possess anti-fungal properties against *Fusarium* (Bertini *et al.*, 2009) and *PR5* (Gao *et al.*, 2013). As *F. graminearum* is known to interfere with the redox state of plant cells through the action of the mycotoxine DON, we also analyzed the expression of peroxidase PRX113-F (***PEROX***), a class III peroxidase that is involved in the deposition of phenolics in

the cell wall (Hiraga *et al.*, 2001) and NADPH oxidase (***NADPHox***) (Desmond *et al.*, 2008). Because cell wall reinforcement is a component of plant defense against fungal pathogens we selected cinnamoyl CoA reductase 3 (***CCR3***) and cinnamyl alcohol dehydrogenase 1 (***CAD1***) as marker genes for lignin biosynthesis (Bi *et al.*, 2011).

qPCR analysis of abovementioned genes revealed that there were no significant differences between the control treatment and the Z-3-HAC pre-treated seedlings in both bioassays (Figure 4-9, Figure 4-10), suggesting that Z-3-HAC does not function as a direct activator of plant defense.

After treatment with MeSA, compared to the control treatment we observed a significant upregulation of *PR1*, *PR4* and *PR5* in both pre-exposed and non pre-exposed seedlings at both time points (Figure 4-10). Additionally, *Perox* showed a significant upregulation in the non pre-exposed seedlings at 24 hai (20 fold, $P<0.05$). Interestingly, at 24 hai after challenge with MeSA, pre-exposed seedlings showed a significant lower upregulation of *PR4* (877 fold vs. 1799 fold, $P<0.05$) and *PR5* (35 fold vs. 68 fold, $P<0.05$), compared to the non pre-exposed seedlings. *LOX1* and *LOX2* expression were not affected by MeSA treatment (Figure 4-10). As SA and JA regulated defense have mainly been reported to act antagonistically (Glazebrook, 2005; Robert-Seilaniantz *et al.*, 2011), we investigated whether a similar or opposite trend was present in pre-exposed and control seedlings after treatment with MeJA. MeJA treatment did not result in significant differences in expression of *PR1* between the different treatments but at 24 hai MeJA did induce a significant upregulation of *PR4*, *PR5* and *Perox* in the pre-exposed treatment, compared to the control (Figure 4-10). Contrary to treatment with MeSA, treatment with MeJA resulted in a significant stronger upregulation in the pre-exposed seedlings, compared to the non pre-exposed seedlings for *PR4* (46 fold vs. 12 fold, $P<0.05$), *PR5* (294 fold vs. 17 fold, $P<0.05$) and *Perox* (259 vs. 31 fold, $P<0.05$ (Figure 4-10). Additionally, *LOX1* was significantly upregulated at 48 hai in the Z-3-HAC + MeJA treatment and the MeJA treatment, compared to the control. Additionally, *LOX1* expression of the Z-3-HAC+MeJA treatment was significantly lower compared to the MeJA treatment. For both the MeSA and MeJA treatment, *PR2*, *NADPHox*, *CCR3*, *CAD1* were not significantly induced (data not shown). Additionally, expression of the biosynthesis genes *PAL* and *ICS* was not affected by MeSA or MeJA treatment (data not shown), suggesting that SA biosynthesis was not affected by these compounds.

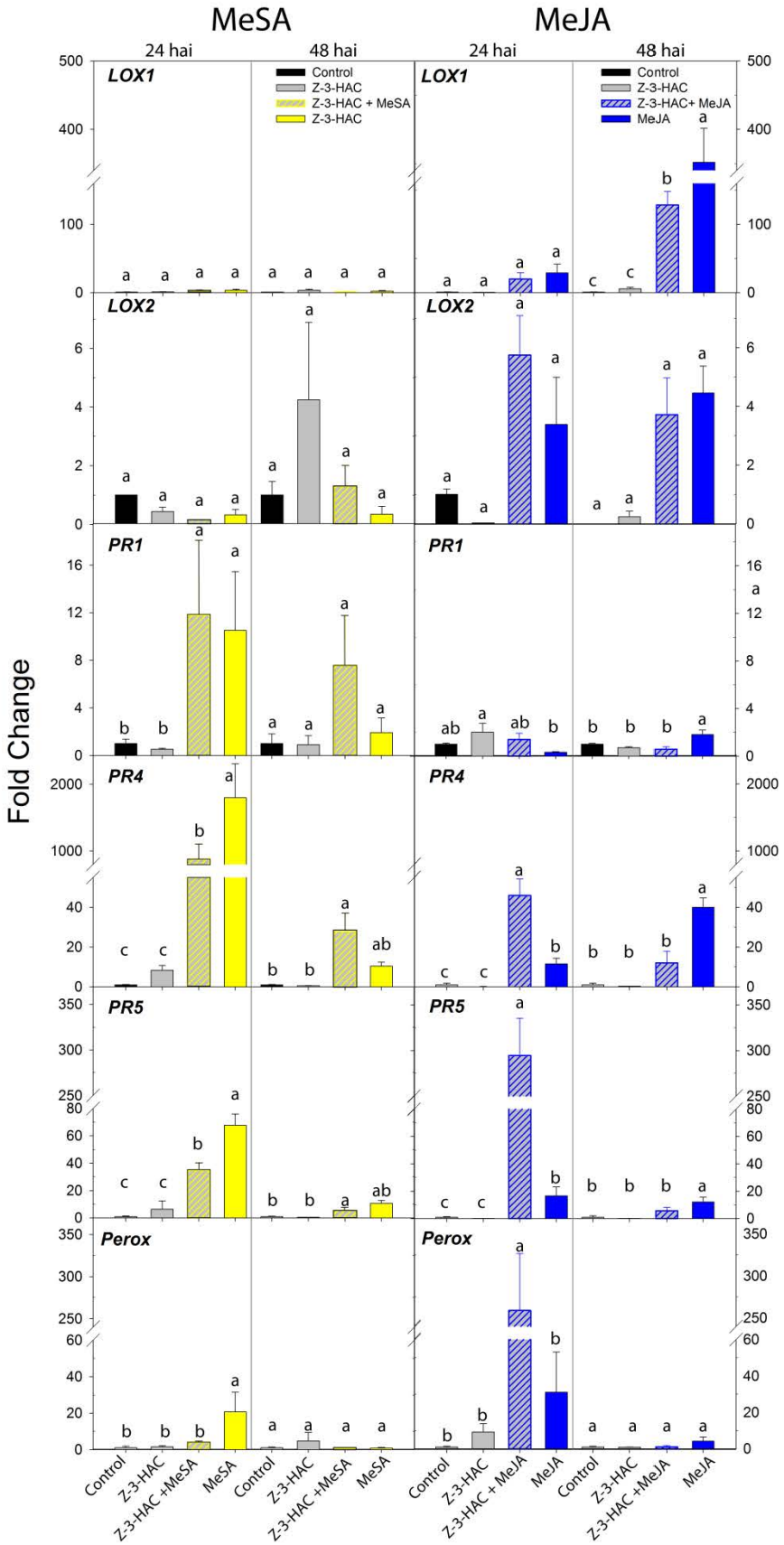


Figure 4-10: Expression profiles of *PR1*, *PR4*, *PR5*, and *PEROX* at 24 and 48 h after challenge with MeSA or MeJA. Leaf sheaths were non-exposed or exposed to Z-3-HAC, followed by a mock, mock, MeSA or MeJA challenge, according to the treatment. At 24 and 48 hai, leaf sheaths were excised and flash frozen in liquid nitrogen for later RNA extraction and qRT-PCR. Data represent means of three biological replicates, each

consisting of four pooled leaf sheaths. Error bars represent SE. Different letters per time point indicate significant differences between the treatments (α= 0.05).

4.4.5 Expression of defense genes upon inoculation with *F. graminearum*

In order to elucidate whether the delay in disease progression of *F. graminearum* in the primed seedlings can be attributed to a stronger activation of JA associated defense through priming by Z-3-HAC, we analyzed the expression of the defense genes (Figure 4-11) upon inoculation with *F. graminearum*.

Both primed and nonprimed seedlings showed a stronger upregulation of *PR1*, *PR4*, *PR5* and *Perox*. Consistent with our results from the MeSA experiment, at 24 hai, we saw a higher upregulation in the nonprimed treatment, compared to the primed treatment of *PR1* (30 fold vs. 10 fold, P<0.05), *PR4* (109 fold vs. 37 fold, P=0.059), *PR5* (90 fold vs. 33 fold, P<0.05) and *Perox* (9 fold vs. 5 fold, P=0.056) (Figure 4-11). At 48 hai the expression pattern followed a similar trend as in MeJA challenged seedlings at 24 hai. Namely, expression of *PR4* (10 fold vs. 1 fold, P<0.05) and *PR5* (5 fold vs. 2 fold, P<0.05) was significantly higher in the primed treatment, compared to the nonprimed treatment (Figure 4-11). *PR2*, *NADPHox*, *CCR3*, *CAD1* were not significantly induced after treatment with Z-3-HAC or inoculation with *F. graminearum* (data not shown).

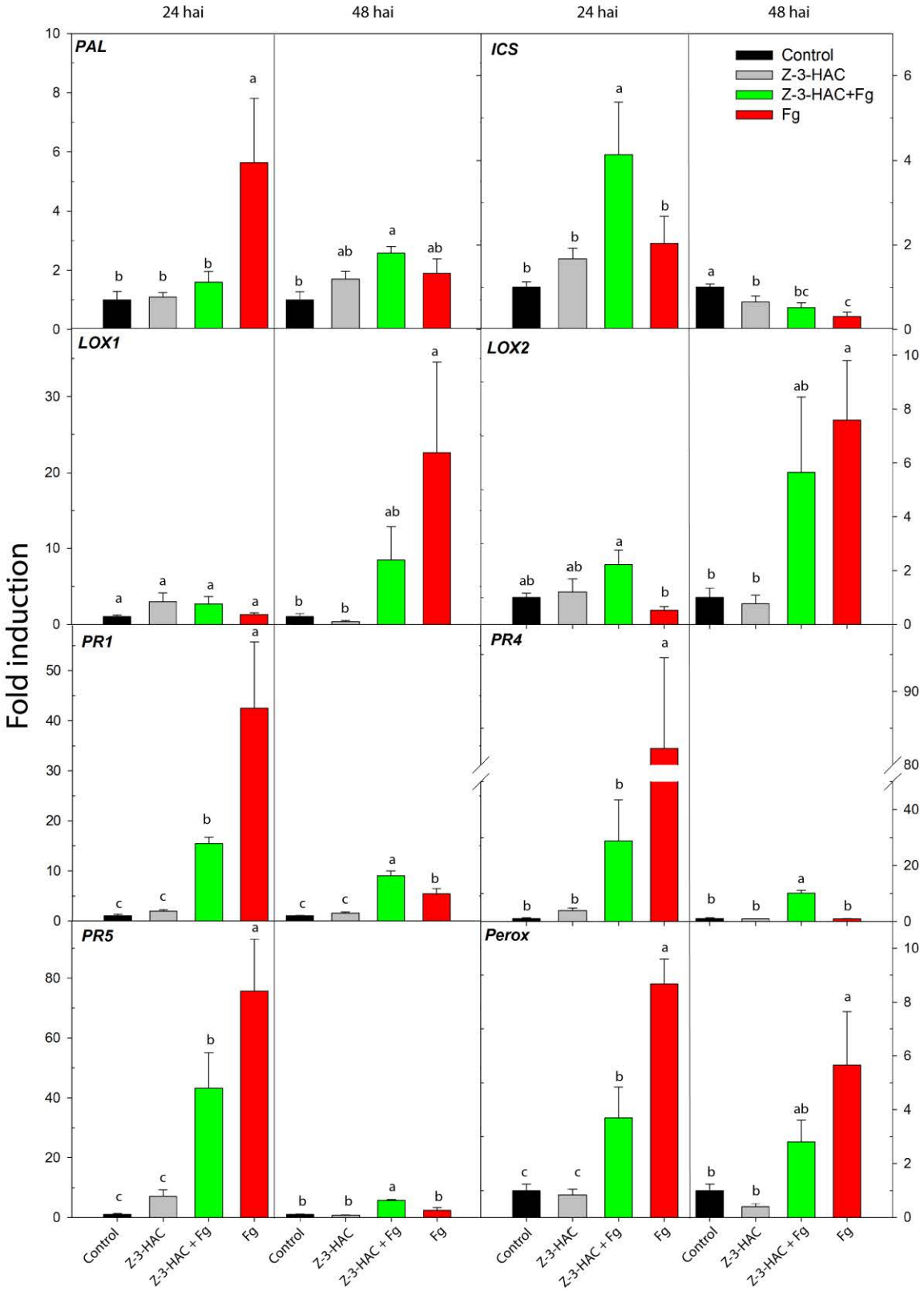


Figure 4-11: Expression profiles of *PAL, ICS, LOX1, LOX2, PR1, PR4, PR5*, and *PEROX* at 24 and 48 h after challenge with a conidia suspension of *F. graminearum*. Leaf sheaths were non-exposed or exposed to Z-3-HAC, followed by a mock, or inoculation with *F. graminearum* (5 x 10^5 conidia ml^{-1}), according to the treatment. At 24 and 48 hai, leaf sheaths were excised and flash frozen in liquid nitrogen for later RNA extraction and qRT-PCR.Data represent means of four biological replicates, each consisting of four pooled leaf sheaths. Error bars represent SE. Different letters per time point indicate significant differences between the treatments (α=0.05). Abbreviations: hai, hours after inoculation.

4.4.6 DON analysis

Owing to its hemibiotrophic character, *F. graminearum* is able to switch from a biotrophic to a necrotrophic lifestyle through the production of DON. As we intended to verify whether the enhanced defense through priming resulted in a higher production of DON, we quantified DON content using U-HPLC-MS.

At 24 hai, DON was present in only one of the four biological replicates of the primed treatment (23.4 ng mg^{-1} plant dry weight) whereas in the nonprimed treatment DON was present in two out of four biological replicates (51.5 ng mg^{-1} plant dry weight) (Figure 4-12). Remarkably, at 48 hai DON content was significantly higher in the primed seedlings than in the nonprimed seedlings (7838.4 vs. 324.5 ng mg^{-1} plant dry weight, $P<0.05$).

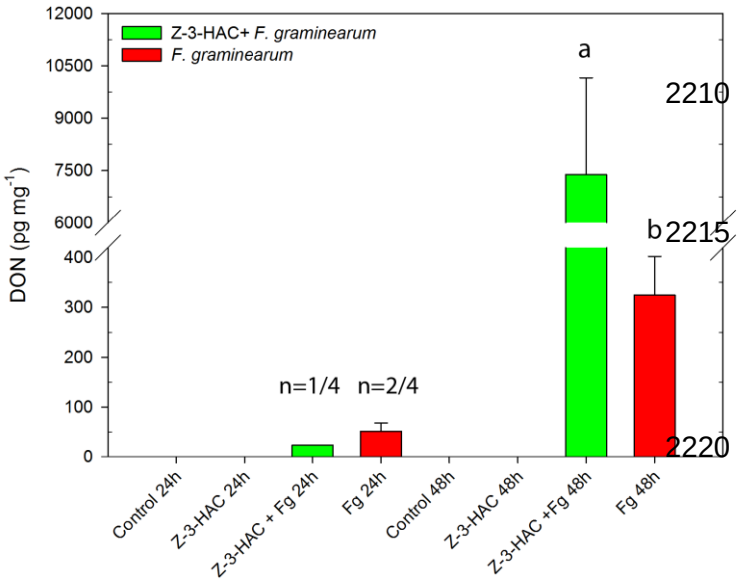


Figure 4-12 DON concentrations (pg mg^{-1} plant dry weight) at 24 and 48 h after challenge with a conidia suspension of *F. graminearum*. Data represent means of four biological replicates, each consisting of six to eight pooled leaf sheaths. Error bars represent SE. Different letters indicate significant differences between the treatments per time point Significant differences were calculated using Student's t-test (α=0.05).

4.5 Discussion

4.5.1 Exposure to Z-3-HAC resulted in enhanced resistance against infection by *F. graminearum*

Priming by GLVs has already been shown for maize (Engelberth *et al.*, 2004), lima bean (Kost & Heil, 2006), poplar (Frost *et al.*, 2008b) and tomato (Finiti *et al.*, 2014). However, despite that wheat is one of the most produced cereals in the world, no studies exist which investigate priming by GLVs in wheat. Additionally, the abovementioned studies mainly investigated the effect of priming by GLVs on defense against herbivore insects, while little research has been done on the potential of GLVs to prime crops against fungal pathogens. Therefore, expanding our knowledge on priming in a plant-fungus interaction is of paramount

importance to obtain a better understanding of the mechanisms and potential of plant priming by GLVs. This study is the first investigating whether GLVs can act as a priming agent for wheat against a fungal infection. We found that exposure of wheat to the GLV Z-3-HAC caused wheat seedlings and ears to become primed against a subsequent infection of *F. graminearum*. We observed for the primed wheat plants a delay in infection in both ears (Figure 4-7A) and seedlings (Figure 4-7B) as well as lower fungal biomass accumulation (Figure 4-7D).

4.5.2 SA and JA contribute to defense against *F. graminearum*

Resistance of wheat against infection by *F. graminearum* has primarily been attributed to SA (Makandar *et al.*, 2012) and JA mediated defense pathways (Li & Yen, 2008; Qi *et al.*, 2012). Because of the hemibiotrophic lifestyle of *F. graminearum,* it can be expected that SA and JA will play a sequential role in the plant defense against *F. graminearum.* This biphasic defense response has already been shown by Ding *et al.* (2011), who reported a peak in SA content followed by a peak in JA content. At gene level, this was confirmed in our study. At 24 hai biosynthesis genes of the SA pathway were more strongly upregulated, indicating an activation of SA signaling (Figure 4-8). However, at 48 hai, the upregulation of SA biosynthesis genes was diminished, and *ICS* expression even downregulated. This coincided with a stronger upregulation at 48 hai of *LOX1* and *LOX2*, which are involved in the biosynthesis of JA. Additionally, pre-exposure to MeSA resulted in smaller lesions, while pre-exposure to MeJA exacerbated the infection by *F graminearum* (Figure 4-7B), suggesting that during the early infection stages, defense is mainly regulated by SA. The negative effect of MeJA pre-exposure on disease development may be attributed to a negative crosstalk between the SA and JA pathway (Robert-Seilaniantz *et al.*, 2011; Pieterse *et al.*, 2012) in which activation of the JA defense pathway would suppress SA defense pathway which is critical in the early defense response against *F. graminearum*. The importance of SA related defense contradicts the study of Li & Yen (2008) who found at 24 hai a significant up-regulation of *LOX* and AOS, an enzyme more upstream of JA biosynthesis. In addition, they reported an upregulation of ethylene responsive genes which led them to conclude that FHB tolerance in wheat is primarily mediated by JA and ethylene signaling; while SA mediated resistance is insignificant. However, it should be remarked here that as the abovementioned study investigated gene expression at 24 hai, it is possible that at this time point, *F. graminearum* had already entered into the necrotrophic phase of its lifecycle (Walter *et al.*, 2010), which consequently would result in augmented JA related defense of the plant. This was confirmed in our experiment. Addition of exogenous MeJA 24 hai rendered the seedlings more resistant (Figure 4-9). These data stress the importance of timing in studying the mechanism of plant-pathogen interaction, especially if the pathogen has a hemibiotrophic

character. The importance of the jasmonate mediated defense against infection by *Fusarium verticillioides* has also been shown for another monocot, maize. Christensen *et al.* (2014) characterized the 9-LOX gene, *ZmLOX12* and showed that mutants exhibited increased susceptibility to infection accompanied by diminished levels of JA. Our results indicate that SA plays an important role during the early stages of infection while JA contributes to resistance during the necrotrophic stage. By precisely switching from SA to JA related defense at the onset of the necrotrophic phase of the pathogen, the plant can defend itself more effectively against infection by *F. graminearum*.

4.5.3 Z-3-HAC primes for a stronger activation of JA related defense

Priming does not directly activate costly defense mechanisms but entails a stronger plant defense upon infection (van Hulten *et al.*, 2006; Conrath, 2009). We did not observe significant effects on gene expression by a pre-exposure to Z-3-HAC (Figure 4-10, Figure 4-11), thus showing that Z-3-HAC did not act as a direct activator of plant defense. Furthermore, as pre-exposure to Z-3-HAC led to enhanced defense after treatment with MeJA or *F. graminearum*, we can conclude that exposure to Z-3-HAC rendered wheat seedlings in a primed state.

Defense against *F. graminearum* is a sequential and meticulously regulated mechanism in which the plant will consecutively employ the SA and JA mediated defense against the biotrophic and necrotrophic phase, respectively, between which a negative crosstalk exists (Glazebrook, 2005; Robert-Seilaniantz *et al.*, 2011).

To elucidate whether the GLV Z-3-HAC targets SA or JA related defense, we analyzed expression of defense genes of primed and nonprimed seedlings after challenge with MeSA and MeJA, respectively. Our results demonstrated that priming of wheat seedlings by Z-3-HAC resulted in a stronger upregulation of *PR4*, *PR5* and *PEROX* after challenge with MeJA, while expression of these genes was suppressed in the primed seedlings after challenge with MeSA, both compared to the nonprimed seedlings (Figure 4-10). These results strongly suggest that Z-3-HAC promotes JA-related defense pathways but antagonizes SA-related immunity. It remains unclear whether Z-3-HAC acts as a direct repressor of SA regulated defense, or as an indirect repressor through a stronger activation of JA related defense. We found evidence that *LOX1* was significantly upregulated upon MeJA treatment, but this upregulation was significantly lower in the primed plants, compared to the non-primed and MeJA-challenged plants (Figure 4-9). Hence, it cannot be ruled out that Z-3-HAC indirectly suppresses SA action by stimulating the JA pathway downstream of JA biosynthesis. Elucidating the exact mechanism(s) by which Z-3-HAC interferes with the SA and JA pathways is a key challenge for future research.

A stronger defense against insects has also been shown in several studies which investigate the effect of BVOC (Karban *et al.*, 2006; Kessler *et al.*, 2006; Ton *et al.*, 2007) and GLVs in particular (Engelberth *et al.*, 2004; Kost & Heil, 2006; Frost *et al.*, 2008b). Engelberth *et al.* (2007) also showed that maize plants which were pre-exposed to Z-3-HAC led to a higher production of JA and 12-oxophytodienoic acid levels after application with caterpillar regurgitant. This is in accordance with a similar study of Ton *et al.* (2007), where maize plants were exposed to the BVOCs of *Spodoptera littoralis*-infested plants seedlings. They reported a higher expression of defense genes after the BVOC-exposed seedlings were subsequently infested with *S. littoralis.* Similar to our study, these BVOCs contained several GLVs and enhanced a specific subset of JA-inducible genes. Therefore, it is conceivable that pre-treatment with GLVs confers to increased resistance against insects and pathogens which are susceptible to JA-related plant defense. Hence, we want to remark here that, since it is known that most plant tissue biting-chewing insects such as caterpillars activate the JA related defense while piercing-sucking herbivores such as aphids induce the SA related defense (Heil, 2008; Smith *et al.*, 2010; Liu *et al.*, 2011), it would be interesting to investigate the priming potential of GLVs against insect herbivores with different modes of feeding.

A study by Scala *et al.* (2013b) investigated the mode of action of another GLV, E-2-HAL, in the defense of *Arabidopsis* against the biotrophic bacteria *Pseudomonas syringae*. They found that plant mutants impaired in the production of GLVs were more resistant against *Pseudomonas* and exhibited higher SA and lower JA levels. They also showed that expression of *MYC2* was not influenced by E-2-HAL, but expression of *ORA59* was. Both genes are important players in the JA signaling pathway, the former contributing to defense against herbivorous insects and the latter promoting resistance to necrotrophic pathogens by integrating the ethylene and jasmonate pathways (Pieterse *et al.*, 2012). Ethylene also plays an important role in the defense of the monocotyl rice against both (hemi)biotrophic and necrotrophic fungi, contributing to both increased resistance or susceptibility (De Vleesschauwer *et al.*, 2013). More research is needed to elucidate whether Z-3-HAC also interacts with the ethylene signaling pathway or other plant defense hormones in wheat.

4.5.4 Nonprimed seedlings exhibit a stronger upregulation of defense genes upon *F. graminearum* inoculation

Our finding that priming by Z-3-HAC activates a stronger JA related defense response concurs with gene expression of seedlings after *F. graminearum* inoculation. At 24 hai we found a significantly stronger up-regulation of defense genes of the nonprimed seedlings compared to the primed seedlings, following the pattern after MeSA challenge. Nevertheless, primed seedlings were able to slow the infection process of *F. graminearum* (Figure 4-7A,B),

suggesting that another mechanism was influenced by Z-3-HAC. At 48 hai the expression pattern of *PR1*, *PR4*, *PR5* and *PEROX* (Figure 4-11) followed the expression pattern of the seedlings at 24 hours after treatment with MeJA (Figure 4-10). Even though at 24 hai expression of these defense genes was higher in the nonprimed seedlings, expression might still have been high enough to contribute to defense in both primed and nonprimed seedlings. However, as *F. graminearum* switched to a necrotrophic phase, the higher gene expression in primed seedlings at 48 hai might have contributed to the enhanced defense of primed seedlings we saw in our infection experiments (Figure 4-7).

The molecular mechanisms for priming remain largely elusive. Recent reviews have attributed the enhanced defense to the accumulation of dormant mitogen-activated protein kinases (MPKs), chromatin modifications, modifications of primary metabolism, accumulation of inactive defense metabolite-conjugates and the activation of a second reactive oxygen species (ROS) burst (Conrath, 2011; Pastor *et al.*, 2013). The direct effect of BVOCs as defense signals in plant-insect interactions has already been shown in different studies (Gatehouse, 2002; Arimura *et al.*, 2005; Kessler *et al.*, 2006). Other studies found minor inductions of gene expression after treatment with GLVs. For example, Bate & Rothstein (1998) exposed *Arabidopsis* seedlings to E-2-HAL. They found induction of PAL, *LOX* and AOS, but no induction of *PR1* and *PR2*. Additionally, the effect of E-2-HAL was only moderate compared to treatment with the volatile MeJA. Engelberth *et al.* (2013) performed a microarray analysis of maize seedlings at 20 min and 60 min after exposure to the GLV Z-3-hexenol (Z-3-HOL). They found a significant expression of genes involved in transcriptional regulation and signaling (AOS, WRKY12 and MYC7, an ortholog of MYC2 in *Arabidopsis*). Furthermore, they suggest that these early regulators serve as a main switch for the subsequent remodeling through the activation of a second-tier level of genes. Thus, these early responses might play a role in the underlying mechanism of defense priming. Contrary to Bate & Rothstein (1998), they found Z-3-HOL to be a more potent inducer of defense genes than MeJA, MeSA and ethylene. In our study, we did not observe a significant difference in gene expression between the control treatment and the wheat seedlings which were exposed to Z-3-HAC but not inoculated at the two time points (Figure 4-10, Figure 4-11). This shows that overnight exposure to Z-3-HAC had no direct effect on gene expression of the tested genes.

4.5.5 *F. graminearum* produces more DON in primed seedlings

Pathogens have evolved different mechanisms to evade or hijack plant defenses in order to successfully infect plant tissue. It has generally been accepted that SA-mediated defense provides protection against biotrophic pathogens and JA-mediated defense against necrotrophic pathogens (Thaler *et al.*, 2012). However, besides SA and JA, plant defense

against pathogens is regulated by an intricate network of different plant hormones between which a complicated crosstalk exists (Lopez *et al.*, 2008; Robert-Seilaniantz *et al.*, 2011; De Vleesschauwer *et al.*, 2013). By manipulating this crosstalk, pathogens can use the host's own defense to their own benefit and successfully infect the plant. This phenomenon has also been described for pathogenic fungi. The necrotrophic fungus *Alternaria alternata* is known to produce different host specific toxins and cause disease on different host plants (Ito *et al.*, 2004). Prasad & Upadhyay (2010) showed that the toxin produced by *Alternaria alternata f. sp. lycopersici* triggers the production of H_2O_2 and ethylene in tomato leaves. The induced production of ethylene is known to further potentiate H_2O_2 production (de Jong *et al.*, 2002), leading to programmed cell death (PCD), thus making the host plant more vulnerable for necrotrophic pathogens. *F. graminearum* also interacts with plant defense through the production of the mycotoxin DON. Namely, after hyphal growth in the apoplast, plants accumulate H_2O_2 to induce programmed cell death in order to counteract the biotrophic phase of *Fusarium.* However, H_2O_2 acts as a signal for *F. graminearum* to produce DON which in turn creates a positive feedback loop leading to increased H_2O_2 and DON production, thus successfully hijacking the plant defense system and clearing the path for the necrotrophic phase of *F. graminearum* (Walter *et al.*, 2010; Audenaert *et al.*, 2013). Because of the health risks associated with DON (Rotter *et al.*, 1996), it is of paramount importance to investigate whether the enhanced plant defense impacts fungal DON production. We found at 48 hai that the DON content in the primed seedlings was up to 22 times higher than in the nonprimed seedlings (Figure 4-12). In contrast, a recent study by Christensen *et al.* (2014) reported on increased growth of the fungus *Fusarium verticillioides* together with increased production of the mycotoxin fumonisin in maize mutants which have been compromised in JA-mediated defense. However, fumonisin does not play a role in the virulence of *F. verticillioides*, contrary to DON in the pathogenicity of *F. graminearum* (Proctor *et al.*, 2002). Thus, the increased fumonisin levels can probably be attributed to larger amounts of fungal mycelium of *F. verticillioides*. We found lower fungal biomass at 48 hai (Figure 4-7D) and a stronger upregulation of defense genes in primed seedlings coinciding with a massive increase in DON (Figure 4-11, Figure 4-12), supporting the hypothesis that *F. graminearum* produced more DON in an attempt to circumvent the enhanced defense. A similar phenomenon has also been reported by Audenaert *et al.* (2010). They showed that treating *F. graminearum* with sub lethal concentrations of fungicides triggered DON biosynthesis. This and our results illustrate that a visible reduction in symptoms not always results in lower DON concentrations. Because of the health risks associated with mycotoxins (Bennett & Klich, 2003), future research efforts should be focused on exploring the impact of enhanced plant defense on mycotoxin production by different fungi.

4.6 Conclusion

In summary, we have found that the GLV Z-3-HAC primes wheat for enhanced defense against the hemibiotrophic fungus *F. graminearum*, resulting in slower disease progress, reduced symptom development, lower fungal growth and higher DON production *in planta*. Furthermore, we show that defense against *F. graminearum* is sequentially regulated by SA and JA and propose a model whereby Z-3-HAC treatment boosts JA-dependent defenses to impede the pathogen during its necrotrophic growth stage.

4.7 Addendum: the effect of Z-3-HAC on plant and fungal growth

4.7.1 The effect of Z-3-HAC on the growth of wheat

As activating defense pathways infers an allocation cost, this often entails a negative effect on growth and yield (see 2.1). To elucidate whether exposure to Z-3-HAC can have negative effects on the growth of wheat, we examined the growth after exposure to different concentrations of Z-3-HAC.

We sterilized wheat seeds in 5% NaOCl for 5 minutes and rinsed the seeds three times with distilled water. Wheat seeds were subsequently placed in test tubes containing 20 mL 1.5 % agar (Oxoid, Hampshire, UK). Wheat seeds germinated and grew in a growth chamber under a 16-h/8-h light/ dark photoperiod at 21±2°C. After 1 week, test tubes were placed in nalophan bags and Z-3-HAC was pipetted on a piece of filter paper inside the bag to reach 0 µM, 0.125 µM, 1 µM and 99 µM inside the different bags. The different concentrations were prepared in MeOH. Bags were closed immediately after the treatment. Each treatment consisted of 6 biological replicates. After 24 hours, the bags were opened and the tubes were placed back in the growth chamber. Leaf length was measured at 0, 24, 48 and 72 hours after treatment. Root length was measured at 72 hours after treatment (Figure 4-13).

At the concentrations of 0.125 and 1 µM, leaf and root length was not significantly different from the control treatment. However, at 99 µM, leaf growth and root growth was significantly lower. After 24h inside the bag, we found no increase in leaf growth. Interestingly, we did observe a small protrusion halfway the leaf stem. Microscopic analysis revealed that inside the leaf sheath of the outer leaf, the new leaf was folded onto itself. This phenomenon is reminiscent of “twisted whorl syndrome” in maize. Twisted whorls can occur with transitions of cool periods with no or slow growth followed by a warm period with a sudden increase in growth. Additionally, herbicides 2,4-Dichlorophenoxyacetic acid (2,4-D) and dicamba which are used in agriculture to control weeds in crop fields can also lead to leafy whorls in maize (Nielsen, 2014). Both 2,4-D and dicamba are synthetic auxins which increase growth. Further research is needed to uncover whether Z-3-HAC induces growth of wheat seedlings at concentrations lower than 99 µM. Furthermore, research on the possible induction of auxin regulated growth after Z-3-HAC exposure might shed light on the observed phenomenon.

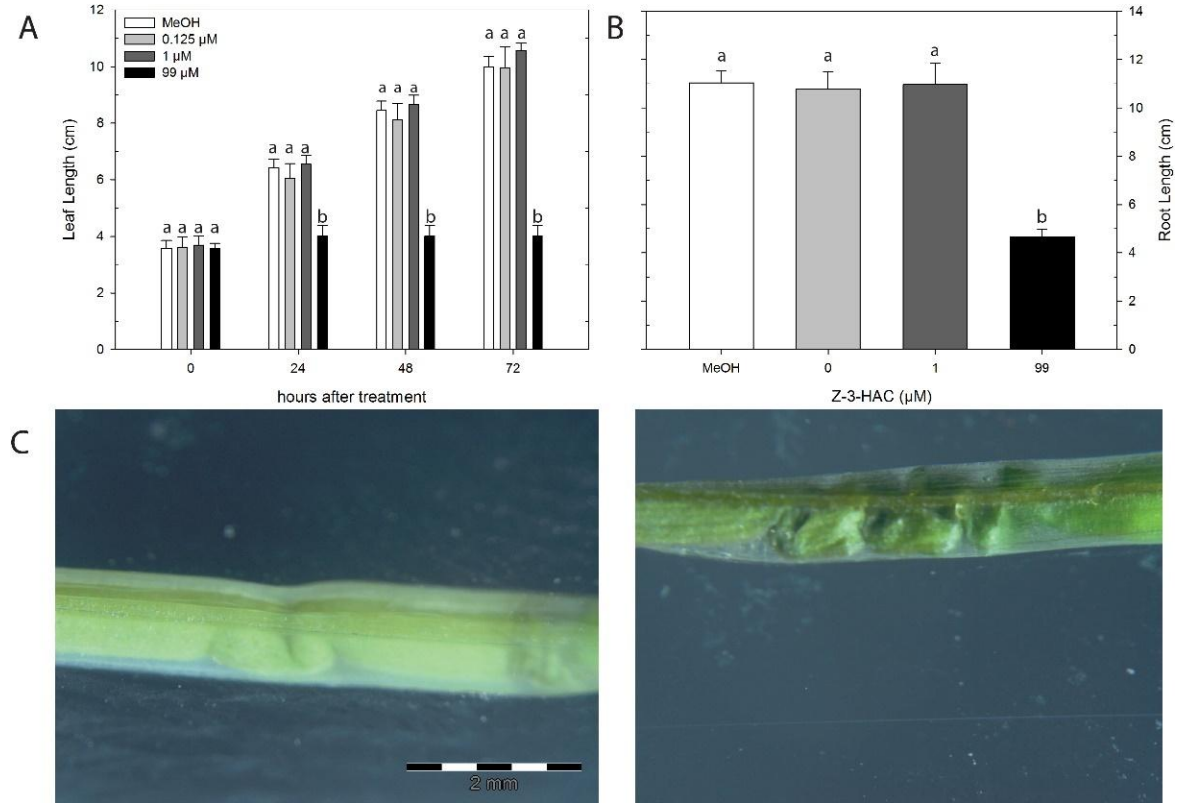


Figure 4-13: The effect of Z-3-HAC on the growth of wheat seedlings. Seedlings were treated with different concentrations of Z-3-HAC (0, 0.125, 1 and 99 µM) via aerial diffusion. A: mean leaf length at different time points; B: root length 72 h after treatment; C: photograph of the leaves of seedlings which were exposed to 99 µM. error bars represent 1 SE. Statistical differences were calculated using a One-way ANOVA with the bonferonni post-hoc test (α = 0.05, n=6)**.**

4.7.2 The effect of Z-3-HAC on the growth of *Fusarium graminearum*

GLVs have already been reported to possess fungicidal activity by inhibiting conidia germination and hyphae growth. For example, Z-3-HAL and E-2-HAL inhibit the growth of *Botrytis cinerea* (Kishimoto *et al.*, 2008). The antifungal activity of E-2-HAL has also been shown against other fungi such as *Alternaria alternata*, *Botrytis cinerea* (Hamilton-Kemp *et al.*, 1992) and *Aspergillus flavus* (De Lucca *et al.*, 2011). However, to our knowledge antifungal effects of Z-3-HAC have not yet been investigated. We inoculated PDA plates with a 10 µL droplet of a spore suspension (10^5 conidia ml^{-1}) of *F. graminearum* on PDA plates and placed them in the same nalophane bags as described previously (**Section 4.7.1**) and exposed them to different concentrations of Z-3-HAC, dissolved in MeOH (0, 0.125, 1, 99 µM). After 4 days, the plates were removed from the bag and the diameter of each fungal colony was measured.

While there were no significant differences for colony diameter between the concentrations of 0, 0.125 and 1 µM Z-3-HAC, treatment with 99 µM Z-3-HAC significantly reduced fungal growth compared to the control treatment (-46%, P<0.001) (Figure 4-14). Furthermore, the colony was highly pigmented at the highest concentration, which is indicative for a higher

production of secondary metabolites (Figure 4-15). Currently, we have no further information on the reason for the delayed growth. A possible explanation may include negative effects on conidia germination and hyphal growth.

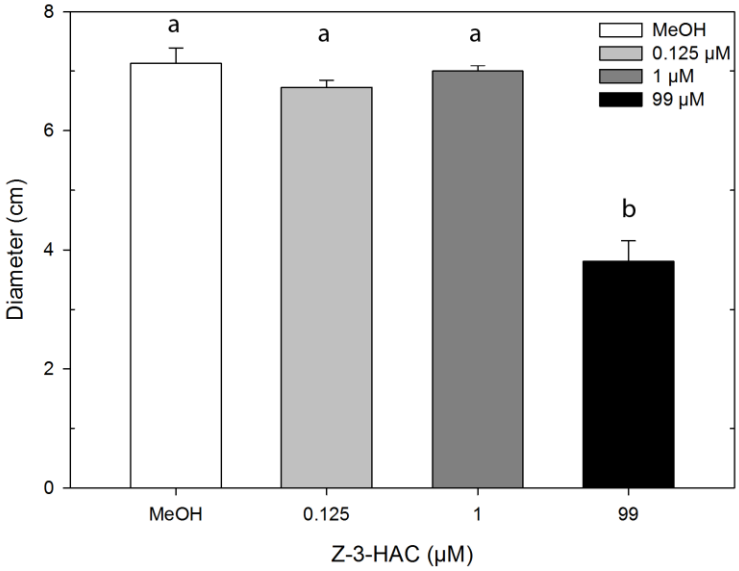


Figure 4-14: Mean diameter of *F. graminearum* after exposure to different concentrations of Z-3-HAC. PDA plates were inoculated with a spore suspension of *F. graminearum*. Plates were placed in the same nalophane bags as in the wheat growth experiments at the same concentrations (0, 0.125, 1 and 99 µM). Fungal growth was measured 96 hours after inoculation. Error bars represent 1 SE. Statistical differences were calculated using a One-way ANOVA test with the bonferonni post-hoc test (α = 0.05, n=6).

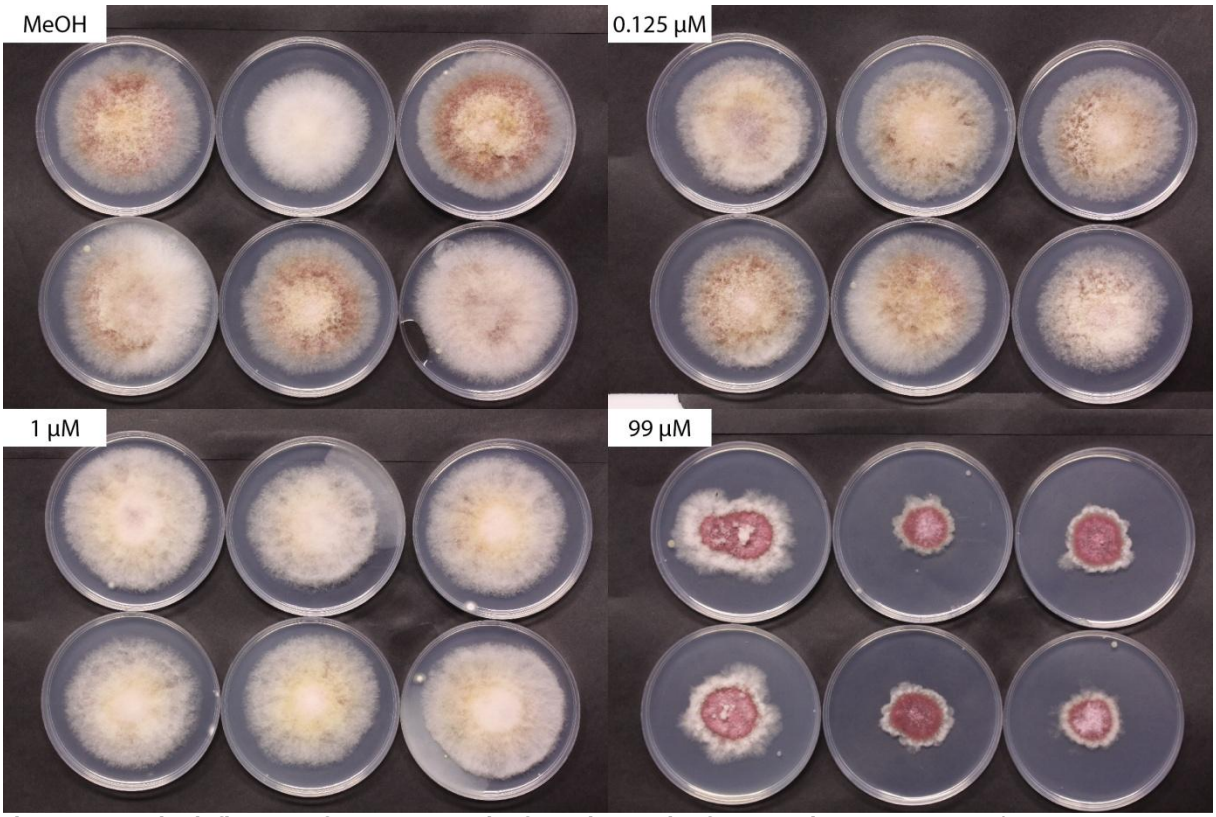


Figure 4-15: The influence of Z-3-HAC on the fungal growth of *F. graminearum.* *F. graminearum* was grown on Petri dishes, containing potato dextrose agar, which were placed inside nalophane bags at different aerial concentration of Z-3-HAC (0, 0.125, 1 and 99 µM)

4.8 Addendum: theoretical concentration of Z-3-HAC in dynamic exposure system.

The negative effect of the high concentration of Z-3-HAC on wheat plants prompts us to investigate whether the overnight exposure to Z-3-HAC is detrimental to the wheat seedlings. Because of the use of an open dynamic instead of a closed loop exposure system (Figure 4-5), the concentration of Z-3-HAC drastically declines after application of Z-3-HAC on the filter paper, thereby exposing the seedlings only for a short period to a high concentration. We calculated the concentration of Z-3-HAC in the gaseous phase by using a mass balance. Figure 4-16 shows the gaseous concentration of Z-3-HAC, MeSA and MeJA If we would assume that for the different assays (Figure 4-6) Z-3-HAC, MeSA and MeJA is immediately vaporized inside the cuvette at 0 min after application, then the maximum concentrations that will be reached at this time point are: 0.11, 0.135 and 0.08 mM, respectively. Because of the continuous supply of fresh air at 600 ml min^{-1} and a corresponding flow out of the cuvette concentrations will drop quite rapidly (Figure 4-16) and will reach concentrations which are in the same range as previously reported values (Table 2-1). It should be noted that this is an overestimation of the aerial concentration as from the moment the compounds were pipetted on the filter paper, the pump was turned on, preventing the compounds from reaching equilibrium.

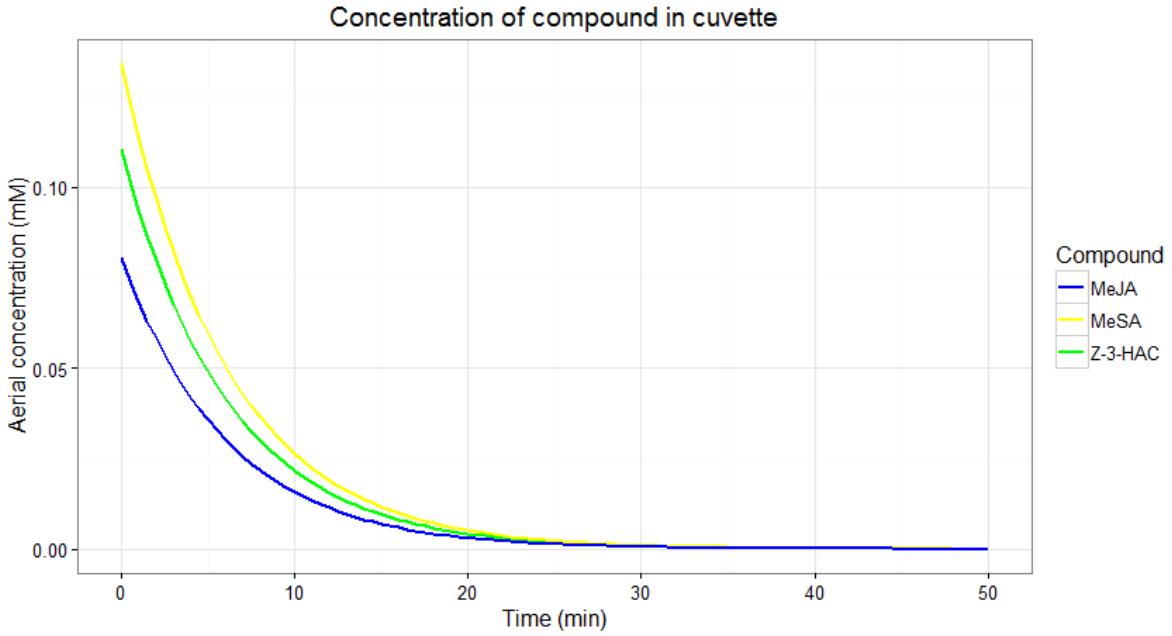


Figure 4-16: Theoretical Z-3-HAC (green), MeSA (yellow), and MeJA (blue) concentration (mM) in the dynamic headspace of the cuvettes. The gaseous concentration of Z-3-HAC was calculated using formulas for stripping columns. Abbreviations: Z-3-HAC, z-3-hexenyl acetate; MeSA, methyl salicylate; MeJA, methyl jasmonate.

Chapter 5 A metabolomics approach to identify compounds involved in Z-3-HAC priming

*Adapted from: Ameye M, Van Meulebroek L, Vanhaecke L, Haesaert G, Smagghe G, Audenaert K, i*n preparation for publication

5.1 Abstract

In the previous chapter, we demonstrated that Z-3-HAC primed wheat defenses against an infection with *F. graminearum* by augmenting JA dependent defenses. However, the mechanism by which the defense is primed remains largely unknown.

In this chapter we attempt to further uncover the mechanism which promotes resistance following Z-3-HAC exposure. By using an untargeted metabolomics approach, we identified metabolites which are significantly increased upon exposure to Z-3-HAC. Remarkably, these metabolites contain fragment ions, which are similar to D-glucose, indicating that Z-3-HAC induces the glycosylation of metabolites. We putatively identified the presence of hexenyl diglycosides, which suggests that aerial Z-3-HAC is metabolized in the leaves by glycosyltransferases and may thus constitute a signaling molecule.

Furthermore, our metabolomics analysis showed that Z-3-HAC exposure also downregulated the biosynthesis of the benzoxazinoid DIMBOA (2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one). We were also interested in the response of a selection of key plant defense hormones, and metabolites of glutamate metabolism after Z-3-HAC treatment. Surprisingly our results showed a strong increase in SA at early time points, whereas JA, IAA and ABA were not influenced. Glutamate, glutamine, L-arginine and GABA were significantly lower in primed seedlings upon inoculation with *F. graminearum*. The findings in this chapter may form the foundation of later research in unraveling the priming and signaling mechanism of Z-3-HAC exposure.

5.2 Introduction

Improved analytical capabilities, lower prices, newly designed bioinformatic tools and data mining strategies have cleared the field for the “omics” era. The triumvirate of genomics, transcriptomics and proteomics has permitted scientists to discover new biochemical pathways and functions in organisms. Also in plant sciences, these techniques have been proven invaluable to progress the fields of biotechnology, metabolic engineering and breeding (Wurtzel & Kutchan, 2016). With the development of tailor-made or species-specific microarrays, transcriptomics has become the most frequently used technique because of its relative low cost and high throughput capabilities. However, both genomics and transcriptomics have their limitations: genes can be subject to epigenetic processes and not all genes are transcribed and translated in functional gene products. So, other omics-techniques such as proteomics and metabolomics are indispensable to bridge the genotype-phenotype gap (Patti *et al.*, 2012; Feussner & Polle, 2015; Hong *et al.*, 2016).

Whereas proteins can be post-translationally modified, metabolites represent intermediary and downstream biochemical products and serve as signatures of metabolic pathways (Patti *et al.*, 2012). Thus, metabolomics in combination with other omics can be used to obtain information leading to the discovery of new genes and pathways (Hong *et al.*, 2016). However, the metabolomics approach in plant physiology is still in its infancy (Creek *et al.*, 2014; Feussner & Polle, 2015) and because of the lack of annotated databases, metabolite identification remains a major limitation for non-targeted metabolomics. Therefore phytometabolome pathways are mostly restricted to genome-reconstructed pathways (Kind *et al.*, 2009; Feussner & Polle, 2015). Due to this bottleneck, many studies have in addition to an untargeted analysis also focused on how plant metabolites from a-priori chosen pathways change in response to pathogen infections (Urano *et al.*, 2009; Ward *et al.*, 2010; Mhlongo *et al.*, 2016). This approach has been termed targeted metabolomics and thus starts from a priori based knowledge. In contrast, untargeted metabolomics consists of an unbiased qualitative and quantitative overview of the metabolites present in an organism (Figure 5-1)(Hall, 2006). It should be noted that a full scan dataset from an untargeted approach, can be used to perform a targeted analysis of a subset of a priori-chosen metabolites (Figure 5-1).

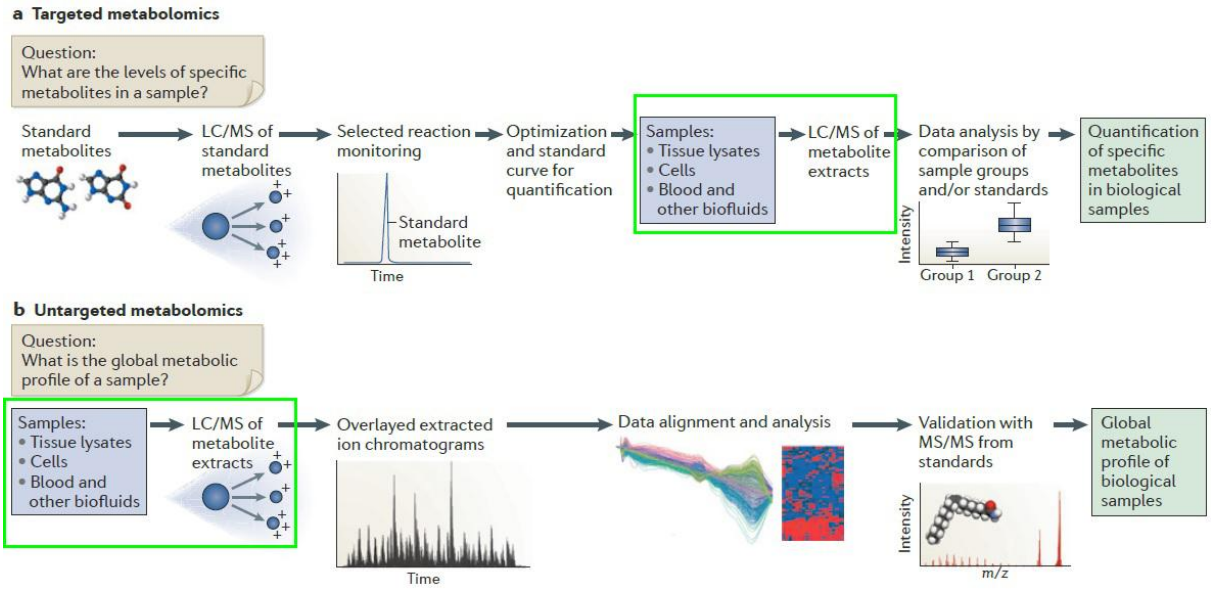


Figure 5-1: Overview of the general workflow of targeted (top) and untargeted metabolomics (bottom). In targeted metabolomics, a priori chosen metabolites are monitored and quantified. An untargeted approach analyses all techniques and using multivariate data analysis techniques, metabolites which are important are withheld and (putatively) identified. Both approaches can use the same dataset of full scan ion chromatograms (green boxes) (Patti *et al.*, 2012).

Techniques are available which reduce the number of metabolites; treatments can be compared and the metabolites can be selected which contribute most to the differences in the metabolome, which are withheld for identification. However, this selection approach, as each other selection criteria, encompasses a risk to overlook important constitutive metabolites (Feussner & Polle, 2015). On the other hand, a non-targeted metabolomics approach provides the benefit of uncovering new biochemical pathways or discovering yet unknown networks which are involved in plant-pathogen interactions.

To obtain a holistic view on plant physiology during biotic stress, future research should utilize multi-omics approaches which combine transcriptomics, proteomics and/or metabolomics, linking changes in the transcriptome to downstream proteins and metabolites (Sana *et al.*, 2010; van de Mortel *et al.*, 2012; Heuberger *et al.*, 2014; Walling & Kaloshian, 2016). It should be noted that the identified genes, transcripts, proteins and metabolites solely based on statistical methods does not necessarily represent important constituents of plant defense. Therefore multi-omics approaches mostly serve as starting points in the search of new building blocks in the defense of plants and further steps (e.g. forward/backward engineering, exposure studies etc.) are necessary to identify and unravel their function.

In the previous chapter we have used RT-qPCR techniques to investigate gene expression at 24 and 48 hai. This chapter includes earlier time points (Figure 5-2) and using a non-targeted approach, we demonstrate that Z-3-HAC exposure and *F. graminearum* inoculation induce large changes in the metabolome of wheat seedlings. We have found that exposure

to Z-3-HAC induces the production of glycosylated compounds, and reduces the level of the benzoxazinoid dihydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA). Furthermore, we have selected different plant defense hormones and amino acids involved in plant defense to get an initial idea whether Z-3-HAC interferes with the defense hormone network and primary N metabolism. The former is known to play a central role in the endurance-evasion hypothesis (Seifi *et al.*, 2013b). Our results show that wheat seedlings treated with Z-3-HAC exhibited a large decrease in L-phenylalanine and concomitant increase in SA 17h after priming with Z-3-HAC. Remarkably, no effect of Z-3-HAC on JA levels was found. Finally, we found a decrease of the metabolites involved in the primary N metabolism in the inoculated tissue. This advocates in favor of a model in which Z-3-HAC exposure leads to a remobilization of N away from the challenged tissue.

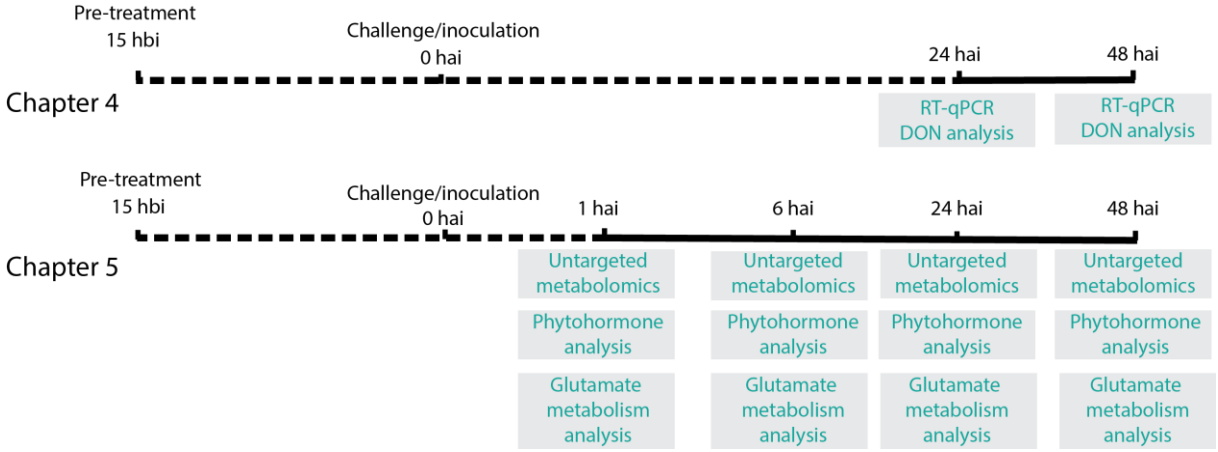


Figure 5-2: Overview of the overlap in timeframes of interest between Chapter 4 and Chapter 5 and the performed molecular analyses used to investigate the Z-3-HAC - wheat - *F. graminearum* interaction. In this chapter we investigated metabolome changes at 1, 6, 24 and 48 hours after inoculation (hai) and investigated changes in the phytohormone balance.

5.3 Materials and Methods

5.3.1 Plant material and sample preparation

The plant material, treatment and inoculated assay are the same as previously described in section 4.3.2, section 4.3.4, and section 4.3.7. In short, the leaf sheaths of two-week-old wheat seedling were inoculated with a conidia suspension (5 x 10^5 conidia ml^{-1}) of a GFP transformant of *F. graminearum* strain 8/1. At 1, 6, 24 and 48 hai leaf sheaths were excised and flash frozen in liquid nitrogen and stored at -80°C for later sample preparation using a solvent extraction (section 4.3.9.1). Each biological replicate consisted of 100 mg of crushed leaf sheaths of different individual seedlings, using liquid nitrogen.

5.3.2 U-HPLC-MS/MS

The U-HPLC-MS system consisted of an Dionex UltiMate 3000 XRS U-HPLC (Thermo Fisher Scientific, San Jose, USA), coupled to an Q-Exactive™ hybrid quadrupole-Orbitrap mass spectrometer (Thermo Fisher Scientific, San Jose, USA) and equipped with a heated electrospray ionization source (HESI-II), operating in both the positive and negative mode (switching polarity mode). Chromatographic separation of the compounds was achieved with a gradient elution program, using a reversed phase Nucleodur Gravity C18 column (1.8 µm, 50 mm × 2.1 mm ID) (Macherey-Nagel, Düren, Germany). The column oven temperature was set at 30 °C. The mobile phase consisted of a binary solvent system: 0.1% formic acid in ultrapure water (solvent A) and methanol (solvent B) at a constant flow rate of 300 µl min^{-1}. A linear gradient profile with the following proportions (v/v) of solvent A was applied: 0–1 min at 98%, 1–2.50 min from 98 to 60%, 2.50–4 min from 60 to 50%, 4–5 min from 50 to 20%, 5–7 min at 20%, 7–7.10 min from 20 to 0%, 7.10–8 min at 0%, 8–8.01 min from 0 to 98%, followed by 2 min of re-equilibration. The instrumental parameters for HESI-II can be found in Van Meulebroek *et al.* (2012).

5.3.3 Non-targeted

5.3.3.1 Chemometric data-analysis

In order to narrow down the number of metabolites which are of interest in our assay, several steps were undertaken. A first step was selecting the metabolites above a given peak intensity threshold and aligning chromatograms to account for inherent variability using the software package Sieve™ (Thermo Fisher Scientific, San José, USA) (1, Figure 5-3). Full-scan data were provided as input and following settings were used: *m/z*-range of 100 - 800 Da, a *m/z* width of 5 ppm, a retention time range of 1.5 - 9.0 min, a peak intensity threshold of 10^6 arbitrary units, a maximum peak width of 0.5 min, and a maximum number of 15,000 frames.

These metabolites were used to construct a predictive model using multivariate data-analysis techniques such as principal component analysis (**PCA**) and orthogonal partial least squares discriminant analysis (**OPLS-DA**) using SIMCA™ 13 software (Umetrics, Malmö, Sweden) (2, Figure 5-3). For the OPLS-DA model, samples were grouped together according to their treatment and time points. In addition, pareto scaling ($1/\sqrt{SD}$, where SD is the standard deviation) was applied to standardize the range of independent X-variables and induce normality.

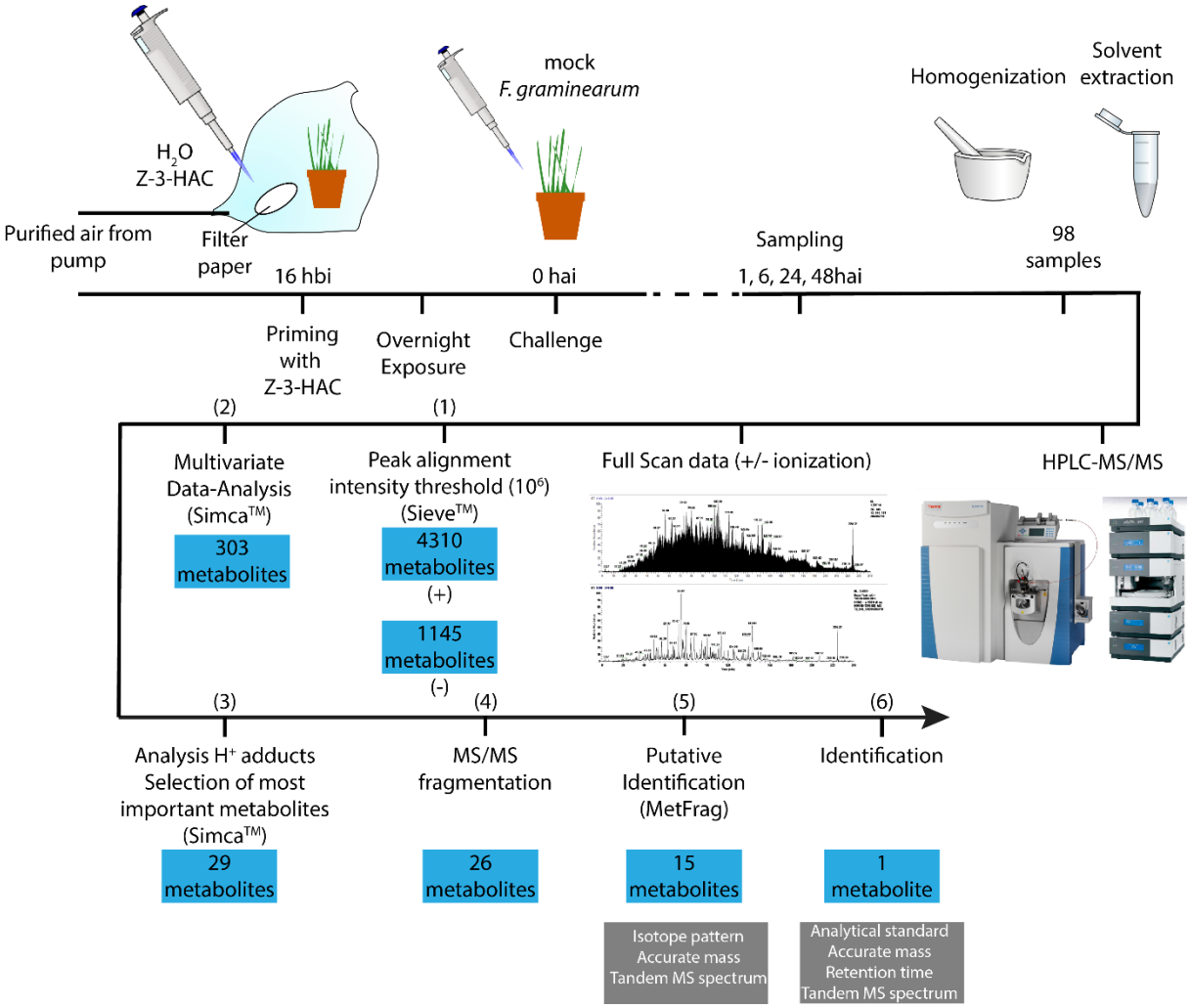


Figure 5-3: Overview of the untargeted metabolomics strategy. In order to identify metabolites which are of importance several selection steps are taken. The numbers in the diagram depict the different steps used, more information on these steps can be found in the text. The number of metabolites in the blue boxes refers to the number of metabolites that were withheld in each selection step in this study. After step 1, only metabolites from the positive ionization mode are displayed.

The model-validity was verified by CV-ANOVA, permutation testing, and considering three model characteristics: **$R^2(X)$** corresponding to the predictive and orthogonal variation in X explained by the model, **$R^2(Y)$** defining the total sum of variation in Y that is explained by the model, and **$Q^2(Y)$** referring to the goodness of prediction calculated by full cross validation. In the following step (3, Figure 5-3), we selected the metabolites which contributed most to the predictability of the model by evaluating the variable in importance projection (**VIP**) scores, loading plots, and S-plots. The VIP scores reflect the importance of an ion towards the predictability of the OPLS model, a VIP score > 1 is generally associated with a significant ion. Loadings plots show the metabolite variability using confidence intervals. Small confidence intervals, which do not overlap with a covariance of value 0, represent more credibility of the selected metabolite towards predictability. S-plots are generated which visualize the correlation of a metabolite towards the model predictive component. Additionally, shared and unique structure (**SUS**)-plots were constructed which are

combination of two two-class models. In our case the two models were *Z-3-HAC vs. Z-3-HAC* + Fg, and *Z-3-HAC+Fg vs. Fg*. The position of a metabolite within a SUS-plot is indicative for its importance towards the different classes. After this selection step, we selected metabolites which were unique (which did not represent isotopes or different adducts of the same metabolite), and were significantly more abundant in a certain treatment for different time points.

5.3.3.2 Identification

The retained selection of metabolites was further analyzed using a combination of different techniques: isotope analysis, accurate mass and MS/MS analysis (4, Figure 5-3). The isotope pattern, the exact mass and the MS/MS pattern were used to putatively identify the metabolites *in silico* using MetFrag (5, Figure 5-3). This web application combines compound database searching with fragmentation prediction (Ruttkies *et al.*, 2016). The public databases used in this study were: ChemSpider (Royal Society of Chemistry), PubChem (National Center for Biotechnology Information), and KEGG (Kyoto Encyclopedia of Genes and Genomes). Putatively identified compounds may be accurately identified using analytical standards by comparing the retention time and MS/MS spectra of unknown metabolites with analytical standards (6, Figure 5-3).

5.3.4 Targeted

For the targeted approach, we used the same full scan dataset that was acquired from the non-targeted approach. We selected several plant defense hormones and metabolites which serve as precursors of major biochemical pathways, known to play a role in the defense against pathogens. These metabolites were supplemented with metabolites involved in the glutamate metabolism of the evasion-endurance model of Seifi *et al.* (2013b). An overview of the set of metabolites of interest is found in Table 5-1.

Table 5-1: Accurate masses, ionization modes and retention times used for the identification of selected metabolites.

Metabolite	Ionization modus	Accurate mass *(m/z)*	Retention Time (min)
Jasmonate (JA)	-	209.11794	5.73
Indole-3-acetic acid (IAA)	+	176.06966	4.51
Gibberellic acid (GA3)	-	345.13461	4.15
Salicylic acid (SA)	-	137.02357	5.05
Abscicic acid (ABA)	-	263.12885	5.25
D6-ABA (Int. Std.)	-	269.1665	5.25
D-Glutamate	+	148.0604	1.15
L-Glutamine	+	147.0764	1.15
L-phenylalanine	+	166.0864	1.51
L-Arginine	+	175.1189	1.26
p-coumaric acid	+	165.05462	4.13
DIMBOA[a]	+	212.05535	3.94
γ-aminobutyric acid	+	104.07065	1.15

[a]DIMBOA was included after putative identification using untargeted metabolomics approach

5.3.5 Data analysis

Data were checked for normality using the Shapiro Wilk test, equality of variances was checked using Levene's test. Statistical comparisons between different treatments were calculated using ANOVA with post-hoc Bonferonni pairs-wise comparison. If variances were unequal, ANOVA with Welch correction and post-hoc Dunnet-T3 test was used (SPSS 22; IBM).

5.4 Results

Two independent non-targeted metabolomics experiments were conducted: one exploratory experiment was performed to assess whether Z-3-HAC priming resulted in changes in the metabolome of wheat at the time points which were investigated in Chapter 4 (24 hai and 48 hai). However, the PCA plots of this introductionary experiment revealed that at 24 h and 48 h after inoculation, there was already a huge difference between the different treatments (Figure 5-10), suggesting that including earlier time points would be more useful in this study. Therefore, in a second experiment we narrowed our time-frame window and included samples from 6 hai. Additionally, more samples were included in the second experiment to reduce variability. Results from this second experiment are discussed in detail below.

5.4.1 Model validation

To obtain a tractable number of metabolites, we employed the strategy depicted in Figure 5-3. The first step consisted of aligning the MS spectrums and retaining the metabolites with an intensity > 10^6 arbitrary units. After this selection step, 4310 metabolites were withheld for the positive ionization mode and 1145 for the negative ionization mode. Secondly, multivariate data analysis methods were used to construct PCA score plots and OPLS-DA prediction models. The PCA scores plots show that samples with the same treatment cluster together and separate from the other treatments (Figure 5-4). This indicates that different metabolites contribute to the separation for each of the different treatments. Total variance explained by the two components is 45.5%, 45%, 55.8%, 65%, and 64.4% for all time points together, 1, 6, 24 and 48 hai, respectively.

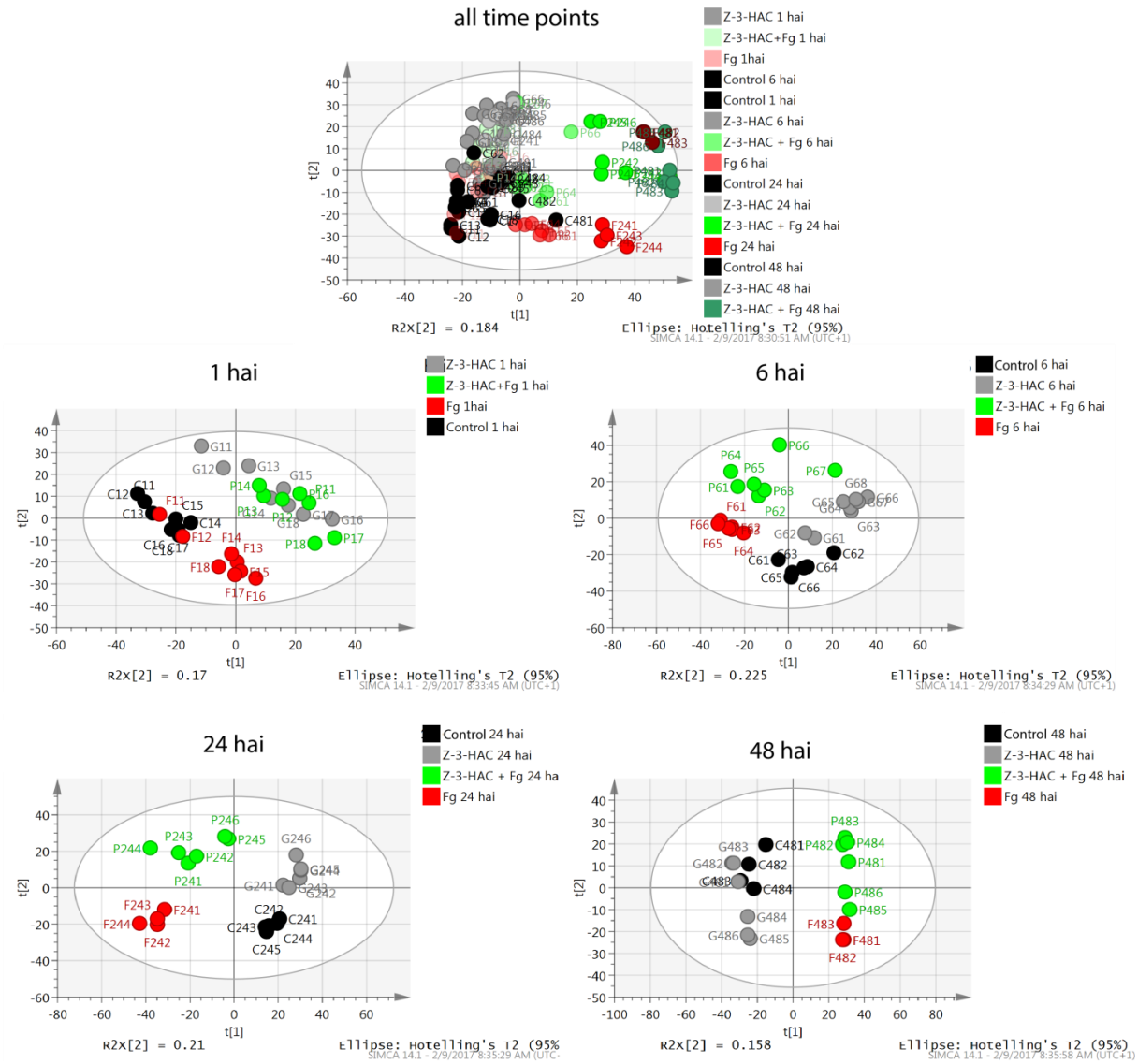


Figure 5-4: Principal Component Analysis score plots of the full scan m/z data for the different time points. Seedlings were placed in bags and a mock treatment or Z-3-HAC was applied on a piece of filter paper according to the experiment. A continuous air flow (600 ml min^{-1}) was supplied to the cuvette to prevent an increase of the relative humidity. Seedlings were kept inside the cuvettes overnight and the day after, seedlings were taken from the cuvettes. Leaf sheaths were subsequently challenged with a mock treatment or a conidia suspension of *F. graminearum.* The following days, leaf sheaths were sampled and prepared for HPLC - MS/MS analysis. The top graph represents the PCA plot with all sample data included. Other PCA plots were constructed with data from a single time point (1, 6, 24, 48 hai). Control: black, Z-3-HAC: gray, Z-3-HAC+Fg: green, Fg: red. The ellipse depicts the Hotteling T2 95% confidence interval. Abbreviations: Z-3-HAC, z-3-hexenyl acetate; Fg, *Fusarium graminearum.*

In the next step, OPLS-DA models were constructed, which permit to identify metabolites which contribute the most to the predictability of the model. According to Triba *et al.* (2015), a good OPLS model has a $Q^2 > 0.5$. In addition, there should not be a large discrepancy between the R^2- and Q^2- values as this would indicate an overfitting of the model. For the positive ionization mode, we saw that R^2 and Q^2 are high for the different time points, indicating a good predictability and fit for the model (Table 5-2). For the negative ionization mode, R^2 values are in general lower, compared to values from the positive ionization. For all models of the negative ionization mode, Q^2 was lower than 0.5. Because of the lack of a

good fit and predictability of the model, we opted to only continue with the model using metabolites of the positive ionization.

Table 5-2: Model characteristics of OPLS-DA models. OPLS-DA models were made for each time-point and for all time points together.

	Ionization	Model characteristics		
Time	**Mode**	$R^2(X)$	$R^2(Y)$	$Q^2(Y)$
All time points	+	0.842	0.561	0.474
	-	0.831	0.427	0.231
1 hai	+	0.775	0.92	0.84
	-	0.832	0.682	0.486
6 hai	+	0.726	0.887	0.808
	-	0.61	0.641	0.489
24 hai	+	0.845	0.98	0.896
	-	0.791	0.809	0.497
48 hai	+	0.70	0.994	0.973
	-	0.54	0.481	0.294

5.4.2 Selection and putative identification of biologically relevant metabolites

Based on the VIP scores, loading plots and S-plots from the OPLS models, we retained 302 metabolites for further analysis. We further narrowed down this selection to a final number of 29 unique metabolites, which did not represent isotopes or different adducts of the same metabolite, and were significantly more abundant in a certain treatment. Two types of patterns emerged: one type in which a metabolite was most abundant in Z-3-HAC, and Z-3-HAC + Fg treatments and another type in which the metabolites were least abundantly present in the Z-3-HAC+Fg treatment (Table 5-3, Figure 5-11). These metabolites were further fragmented using tandem MS. Unfortunately, three metabolites could not be found again in the sample and could not be fragmented.

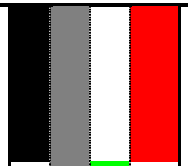
The retained selection of metabolites, were putatively identified using a combination of different techniques: isotope analysis, accurate mass and MS/MS analysis. This was used to identify the metabolites using MetFrag in combination with the public libraries ChemSpider, PubChem and KEGG. However, not all metabolites got a hit, which may be attributed to the selection of an isotope in a previous selection step instead of the C12 mother ion.

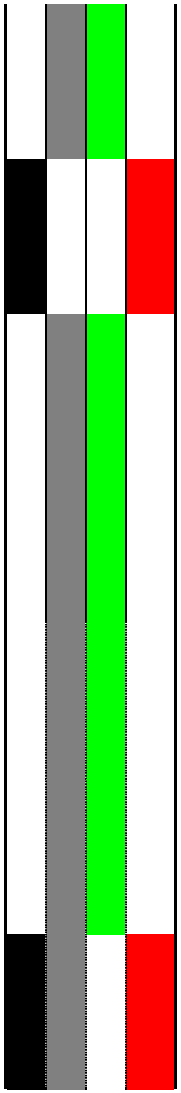
Metabolites which were not putatively identified using MZmine or MetFrag were excluded from Table 5-3.

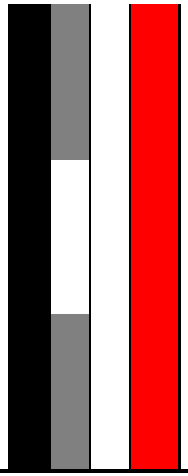
Remarkably, metabolites which were most abundantly present in Z-3-HAC-treated plants were numerously identified as glycosylated compounds using MetFrag. Using the online platform MZcloud™ which aggregates experimental tandem MS data, we were able to putatively identify a large fraction of the fragment ions to originate from D-glucose[2]. Using an analytical standard, we analyzed the MS/MS pattern of D-glucose and found several matching fragments with the glycosylated compounds, demonstrating the presence of a glucose group on the metabolites (Table 5-4). Besides the fragments which were shared with fragment ions of D-glucose, other fragment ions were also shared between metabolites (89.06017, 116.0708, 121.065, 151.0753, and 301.125). These data strongly indicate that after exposure to Z-3-HAC, glycosylated compounds were formed. The presence of shared fragment ions, which are not unique for D-glucose may suggest that some metabolites from Table 5-4 share the same molecular backbone and may thus originate from the same, yet unknown, pathway.

[2] MZcloud reference number 6210

Table 5-3: Overview of metabolite ions which contribute the most to the predictability of the OPLS-DA model. Metabolites are listed with their metabolite ID, mass over charge ratio (m/z) of the positive ionization mode and retention time. Predicted elemental formulas are calculated using the software package MZmine 2™. Treatments in which the metabolites had the highest abundance are shown using the colour code previously depicted (**Table 4-2**): black: control, grey: Z-3-HAC; green: Z-3-HAC+F. *graminearum* inoculation; red: *F. graminearum* inoculation. If a color field is left blank, the metabolite abundance was lowest for that treatment. In-silico identification was performed using the web application MetFrag beta. The number of the matching fragments and m/z ions from the MS/MS analysis are listed of the candidate metabolite with the highest score using the public library ChemSpider.

Metabolite ID	m/z $[M+H]^+$	RT (min)	Predicted elemental formula	Highest abundance	Matching fragments	Matching Fragment ions	Candidate metabolite with highest score
1022	212.05525	4.27	$C_9H_9N_1O_5$		15/20	195.03,194.04456,177.04222,166.04965, 165.04221,151.02621,149.04698,141.05 467,138.05478,134.02368,123.04408,12 2.06007,120.04449,107.0386,95.0495,95 .03693	2,4-Dihydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA)
1510	253.12785	1.47	$C_{10}H_{20}O_7$		17/20	145.04927,127.03896,115.03916,99.044 5,97.02876,91.03941,87.04455,85.0289, 81.0341,73.06543,73.029,71.04977,69.0 3416,61.02915,57.03426,55.0550,55.018 56	4-Hydroxy-2-butanyl beta-D-galactopyranoside
2193	301.1256	3.69	$C_{14}H_{20}O_7$		4/16	121.06495, 97.02871,89.06017,85.0289	Salidroside (glucopyranoside)
2354	317.09949	3.43	$C_{13}H_{12}N_6O_4$		13/19	245.0797,203.07018,179.07027,175.075 44,165.05443,161.0594,151.0753,149.05 835,137.05963,123.04363,97.02874,85.0 2897, 69.03423,	Ethyl 2-methyl-6-(4-nitro-1H-pyrazol-1-yl)pyrazolo[1,5-a]pyrimidine-3-carboxylate
2778	349.14898	5.03	$C_{15}H_{24}O_9$		17/20	151,07527,141.09085,139,07526,127.03 9,123.08051,111.08064,95.08595,93.069 88,85.02891,83.08605,8.04968,81.07043 ,81.03403,71.0498,69.03419,57.03429,5 5.05507	Leonuridine (glucopyranoside compound)

3194	388.1293	3.9	$C_{15}H_{22}N_3O_7P$		11/18	344.13295,222.07582,208.06042,177.05467,164.03421,85.02898	2-Oxo-N-(2-[(6-phenyl-3-pyridazinyl)oxy]ethyl)-2H-chromene-3-carboxamide
3435	412.06369	4.06	$C_{20}H_{13}NO_9$		4/20	177.05443,127.03905,85.02897,69.03412	4,4'-[(5-Hydroxy-4-nitro-1,3-phenylene)bis(oxy)]dibenzoic acid
3444	412.2171	4.34	$C_{19}H_{25}N_9O_2$		8/26	263.1484,245,13797,233.13925,163.05996,150.0774,145.04958,139.07557,83.08553,	1-[7-(6-Amino-9H-purin-9-yl)heptyl]-3,7-dimethyl-3,7-dihydro-1H-purine-2,6-dione (Z)-3-hexenyl-O-α-L-arabinopyranosyl-(1,6)-β-D-glucopyranoside*
3567	424.21723	5.13	$C_{18}H_{33}NO_{10}$		14/20	187.059898,163.05981,145.04941,103.03929,99.0444,89.06019,87.0447,85.02892,73.02909,71.0498,69.03416,61.02913	6-O-[2,4-Dideoxy-4-(methyl([(2-methyl-2-propanyl)oxy]carbonyl)amino)-alpha-L-threo-pentopyranosyl]-4-O-methyl-D-glucopyranose
3666	445.14753	5.18	$C_{23}H_{24}O_9$		10/20	133.0858,127.03874,111.04445,103.03945,99.04405,89.06015,87.04446,85.0284,73.02904,69.03414,	(6S)-2,6-Anhydro-1-deoxy-6-[5-hydroxy-7-methoxy-3-(4-methoxyphenyl)-4-oxo-4H-chromen-6-yl]-D-erythro-hexitol
3724	456.2094	4.31	$C_{19}H_{29}N_5O_8$		7/20	302.13635,127.03908,113.07118,85.02898,73.02911,70.06585,69.03424,	Pro-pro-asn-glu
3758	461.23476	6.03	$C_{22}H_{36}O_{10}$		17/20	189.12703,171.11655,161.13219,147.11661,145.10094,143.08533,133.10106,119.08562,107.08572,105.07012,95.04945,93.07027,91.05464,81.0704,79.05477,67.05491	1-Acetoxy-1-(3-([6-(3,5-dimethylbenzyl)-5-isopropyl-2,4-dioxo-3,4-dihydro-1(2H)-pyrimidinyl]methoxy)propyl)urea

3770	464.24035	6.06	$C_{24}H_{29}N_7O_3$		9/20	184.07312,93.07026,91.05455,86.09692, 81.07027,79.05463,71.07367,67.05494,5 8.06632	1-[4-(1-Cyclopropyl-1H-tetrazol-5-yl)phenyl]-3-(3-[2-(4-morpholinyl)ethoxy]benzyl)urea
4208	609.11274	3.99	$C_{32}H_{20}N_2O_{11}$		4/17	465.07123,447.06085, 419.06201,398.06622	2,2'-(Oxybis[(1,3-dioxo-1,3-dihydro-2H-isoindole-5,2-diyl)-4,1-phenyleneoxy])diacetic acid
4302	771.16467	4.18	$C_{26}H_{32}N_{10}O_{14}P_2$		4/20	255.02934,194.04507,132.04434,	3'-O-[([2-(6-Amino-7H-purin-7-yl)ethoxy]methyl)(hydroxy)phosphoryl]-5'-O-[([(2Z)-2-(3,4-dimethoxy-5-oxo-2(5H)-furanylidene)ethyl]oxy)(hydroxy)phosphoryl]adenosine

* metabolite 3444 was putatively identified as (Z)-3-hexenyl-O-α-L-arabinopyranosyl-(1,6)-β-D-glucopyranoside based on the fragmentation pattern reported by Sugimoto *et al*. (2014).

5.4.3 Identification

Of the selected compounds, only metabolite 1022, which was putatively identified as 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA), was commercially available as analytical grade standard (Santa-Cruz Biotechnology,Dallas, TX, USA). We performed a HPLC-MS/MS analysis using the same settings as described in section 5.3.2. Based on the retention time, exact mass and MS/MS spectra of both the metabolite 1022 and analytical standard DIMBOA, we were able to identify metabolite 1022 to be DIMBOA (Figure 5-5).

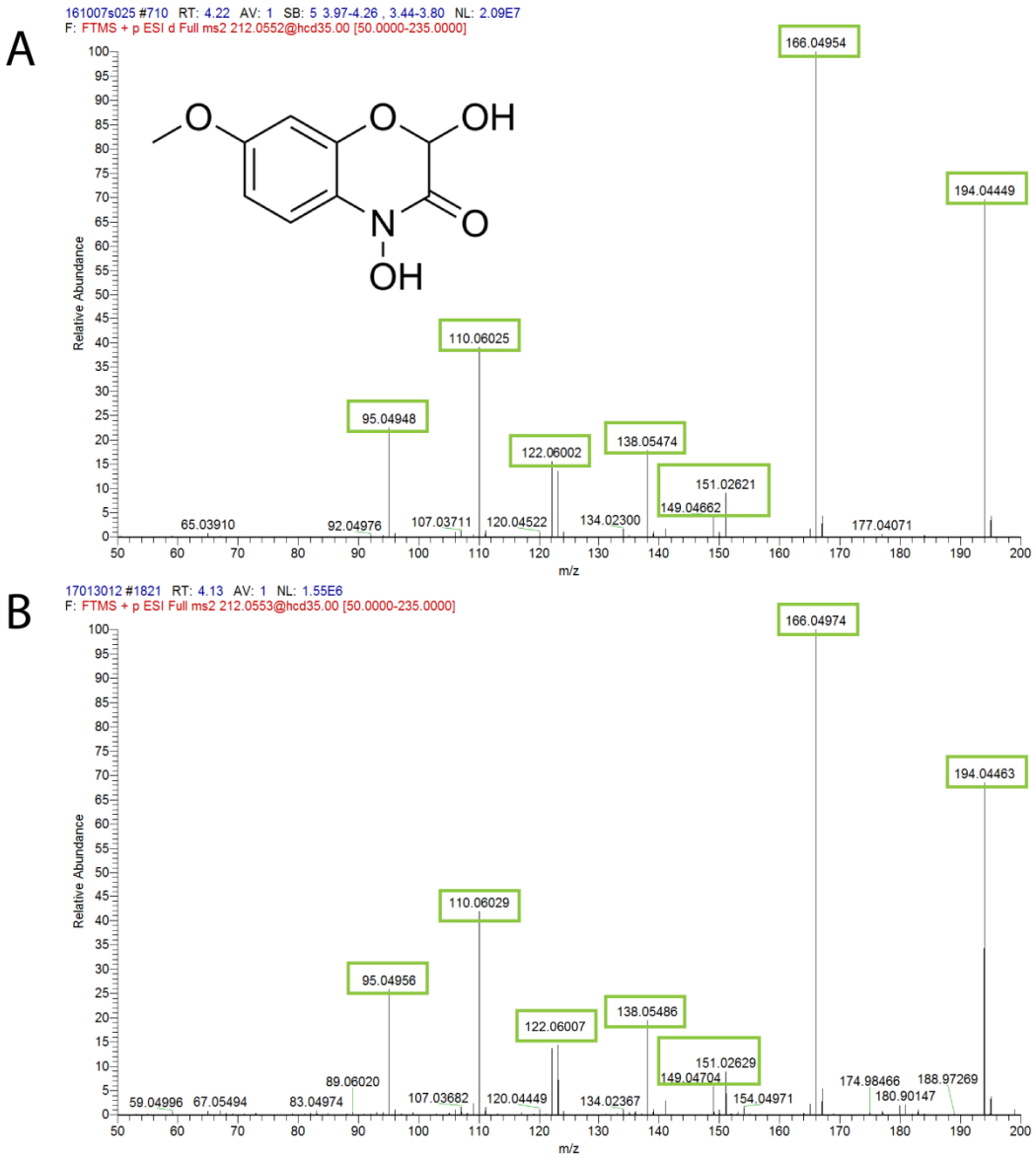


Figure 5-5: The MS/MS spectrum of metabolite 1022 (A) is identical to the MS/MS spectrum of an analytical standard of DIMBOA (B). A: the top graph shows the MS/MS spectrum of metabolite 1022, from a plant sample. B: the bottom graph is the MS/MS spectrum of an analytical standard of DIMBOA (Santa Cruz Biotechnology, Inc., Dallas, TX, USA). The m/z fragments which are identical between the two spectra are depicted with a green box.

DIMBOA belongs to the group of the benzoxazinoids. Benzoxazinoid biosynthesis starts from indole and results in the formation of glycosylated compounds which are stored in the

vacuole. Following cellular damage, these compounds are released and converted by β-glucosidases, which are located on the plastids, to the more toxic aglucons of which DIMBOA is the most abundant (Von Rad *et al.*, 2001; Frey *et al.*, 2009; Niemeyer, 2009).

We saw a significant reduction of DIMBOA for Z-3-HAC treated seedlings at 1 and 6 hai, compared to the control treatment. Furthermore, DIMBOA levels were the lowest for the Z-3-HAC+*Fg* treatment (Figure 5-6).

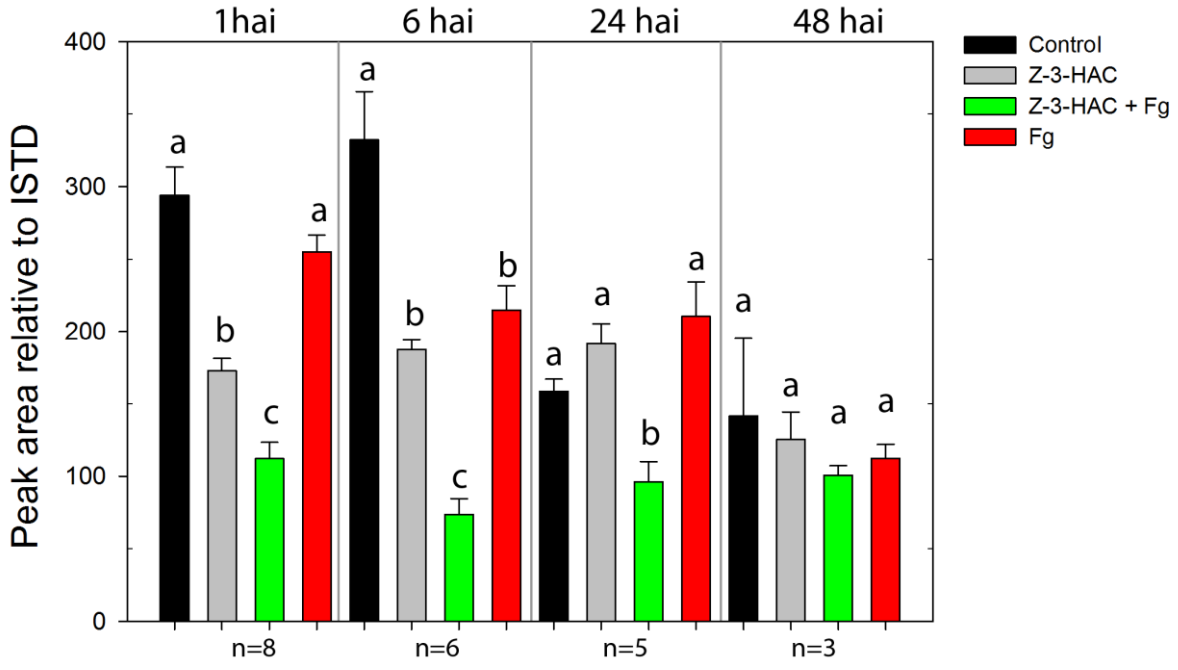


Figure 5-6: Amount of DIMBOA is lower in Z-3-HAC-treated leaves after inoculation with *F. graminearum.* Peak area relative to the internal standard (ISTD) is shown. As internal standard, a deuterium labelled analytical standard of 100 pg µl^{-1} d_6-abscicic acid (OlChemIm, Olomouc, Czech Republic) was used. . The number of biological replicates per treatment are shown beneath each bar. Each biological replicate consists of 100 mg fresh weight of pooled leaf sheats. Error bars represent SE. Significance of differences was calculated using one-way ANOVA with a post-hoc bonferonni test.

Additionally, we were able to identify another metabolite (3444). Sugimoto *et al.* (2014) exposed 24 different plants to Z-3-HOL and found increased production of the diglycoside HexVic ((Z)-3-hexenyl-O-α-L-arabinopyranosyl-(1,6)- β-D-glucopyranoside)(Figure 5-7) with an m/z value of 412.21765 +/- 10 ppm ($[M+NH_4]^+$) . Similarly, we determined metabolite 3444 to be only produced in Z-3-HAC treated plants and which has an m/z value of 412.2171 and contains mass fragments which are linked to sugar groups (Table 5-3, Table 5-4). Additionally, the MS/MS spectrum from HexVic contain m/z ions of 263, 295, 233, 245 in descending order of abundance (Koichi Sugimoto, personal communication, February 25, 2017), which are also abundant in the MS/MS spectrum of metabolite 3444 (Table 5-4). Together, these data indicate that metabolite 3444 most likely can be identified as HexVic.

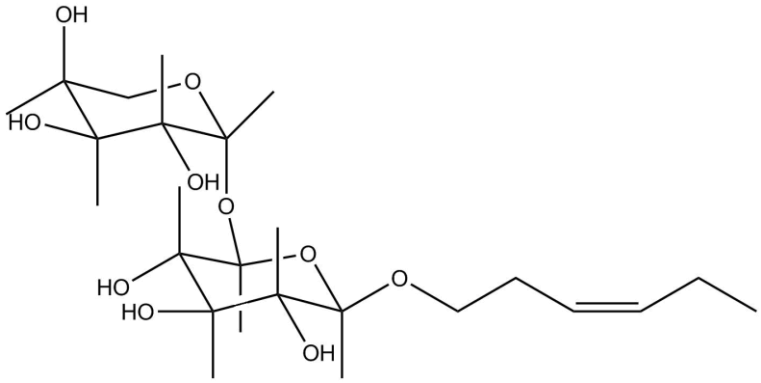


Figure 5-7: Visual representation of HexVic ((Z)-3-hexenyl-O-α-L-arabinopyranosyl-(1,6)- β-D-glucopyranoside). Adapted from Sugimoto *et al.* (2014)

5.4.4 Targeted metabolomics

5.4.4.1 Phytohormone analysis

In Chapter 4, we analyzed the biosynthesis genes of SA (*PAL*, *ICS*) and JA (*LOX1*,*2*) (Figure 4-11). However, these indirectly give an indication of SA- and JA levels in the seedlings. To get an insight into the effect of priming and subsequent inoculation with *F. graminearum* on the plant hormone balance, we measured plant hormones at different time points after inoculation with *F. graminearum*. SA, JA, ABA, and IAA, were measured and quantified using HPLC-MS. GA3 was also measured but did not exceed the limit of quantification and was not included in Figure 5-8.

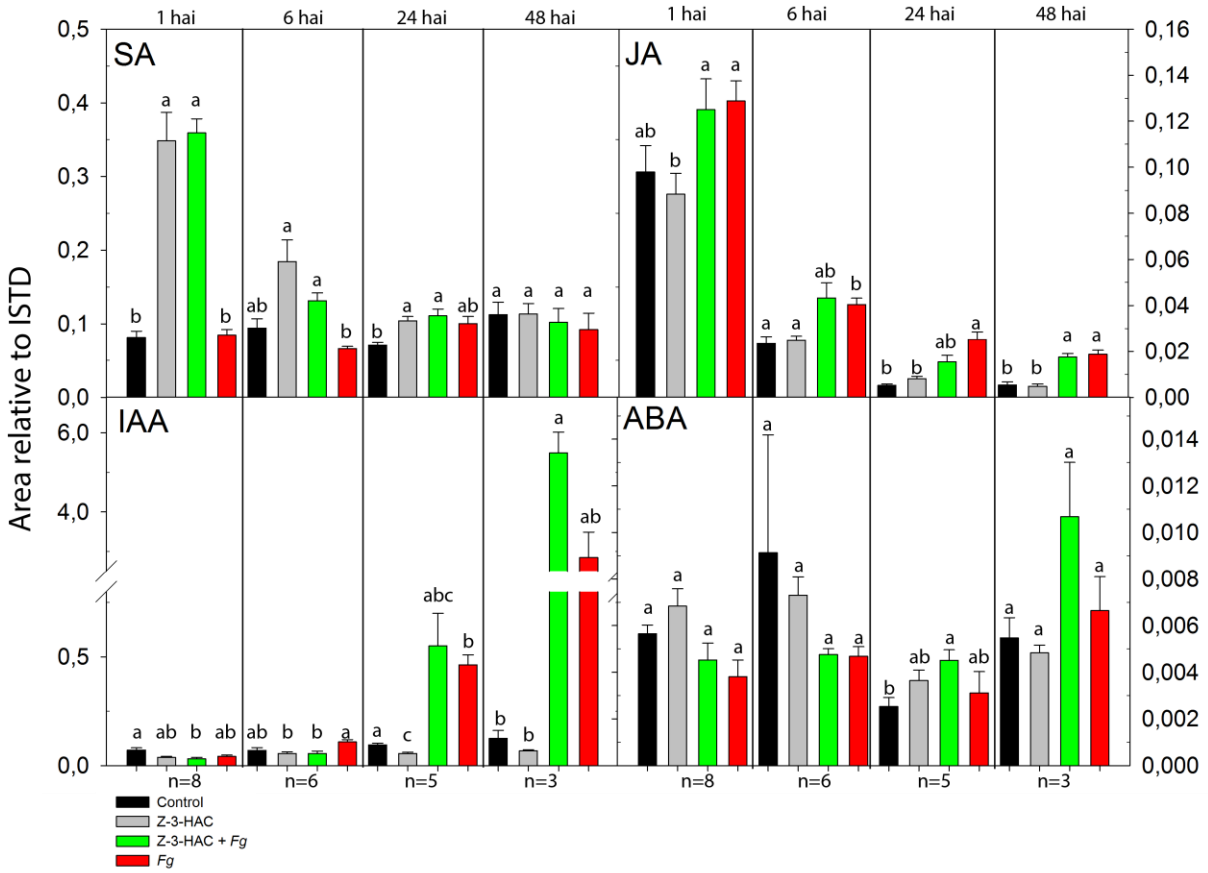


Figure 5-8: Plant hormone analysis (area relative to the internal standard) of seedlings after inoculation with a spore suspension of *F. graminearum*. Peak area relative to the internal standard (ISTD) is shown. As internal standard, a deuterium labelled analytical standard of 100 pg µl^{-1} d_6-abscicic acid (OlChemIm, Olomouc, Czech Republic) was used. The number of biological replicates per treatment are shown beneath each bar. Each biological replicate consists of 100 mg fresh weight of pooled leaf sheats. The number of biological repeats per bar are shown beneath each bar. Abbreviations: salicylic acid (SA), jasmonic acid (JA), indol-3-acetic acid (IAA), and abscicic acid (ABA). Significant differences between treatments are depicted with different letters for each time point. Error bars represent SE. Significance of differences was calculated using one-way ANOVA with Welch correction for unequal variances and Dunnett's T3 post hoc test (α=0.05).

For SA, we observed a three-fold increase in seedlings which had been treated with Z-3-HAC at 1 hai ($P<0.01$). Also, at later time points SA content was higher in Z-3-HAC and Z-3-HAC+ *Fg* treated seedlings, but this difference was only significant at 24 hai, compared to the control (Figure 5-8). JA was consistently higher in the treatments which had been inoculated with *F. graminearum*. However, no effect of Z-3-HAC was apparent. There was a strong increase in IAA levels at later time points in *F. graminearum* inoculated seedlings, but only a minor, non significant effect of Z-3-HAC treatment on IAA could be seen. For ABA, no clear trends were observed (Figure 5-8).

5.4.4.2 Amino acid analysis

As mentioned in section 3.6, GLVs might influence primary C/N metabolism and promote an evasion or endurance strategy by interfering with the glutamate metabolism (Seifi *et al.*, 2013b). We selected glutamate, glutamine, L-arginine and γ-aminobutyric acid (**GABA**).

For none of the metabolites at any time point, there was a difference between control and *F. graminearum* inoculated seedlings (Figure 5-9). On the other hand, at 6 hai we observed a decline in glutamate, glutamine, L-arginine and GABA in Z-3-HAC-, and Z-3-HAC+*Fg*-treated seedlings, of which the latter treatment showed the largest decrease. It should be noted that seedlings were exposed to Z-3-HAC 15 hours prior to the inoculation. Hence, possible effects on Z-3-HAC treated seedlings at 1 hai were 16 hours after treatments with Z-3-HAC.

We also selected L-phenylalanine (**Phe**), which is the precursor of the phenylpropanoid pathway, together with p-coumaric acid. For Phe, we observed in Z-3-HAC-treated seedlings a significant decrease, compared to control and *Fg* inoculated seedlings (Figure 5-9). P-coumaric acid was not significantly different at the early time points but did increase at 24 and 48 hai in *Fg* inoculated seedlings.

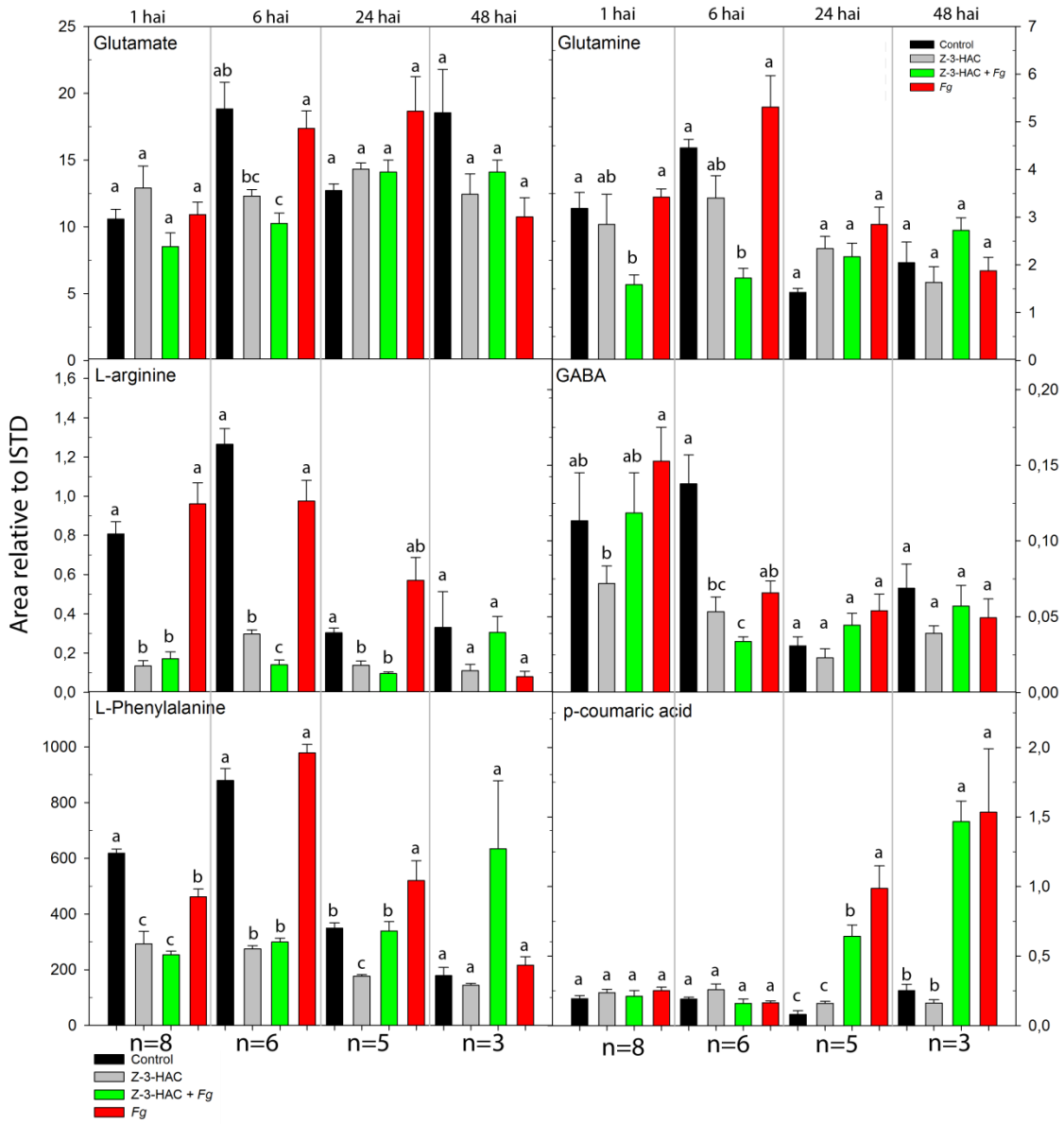


Figure 5-9: Metabolites involved in glutamate metabolism show a lower level in the primed treatment. Z-3-HAC exposure leads to lower levels of L-phenylalanine, but has no effect on p-coumaric acid. Peak area relative to the internal standard (ISTD) is shown. As internal standard, a deuterium labelled analytical standard of 100 pg µl^{-1} d_6-abscicic acid (OlChemIm, Olomouc, Czech Republic) was used. The number of biological replicates per treatment are shown beneath each bar. Each biological replicate consists of 100 mg fresh weight of pooled leaf sheats. Error bars represent SE. Significance of differences was calculated using one-way ANOVA with a post-hoc bonferonni test.

5.5 Discussion

5.5.1 Z-3-HAC induces glycosylation of metabolites

Glycosyltransferases (**GTs**) play an important role in the biosynthesis and maintenance of the cell wall (Scheible & Pauly, 2004). Furthermore, GT's are also implicated in plant defense by detoxifying xenobiotic compounds, and stabilizing and increasing the solubility of plant defensive compounds such as phyto-anticipins, plant defense hormones and its precursors.

After glycosylation, these compounds can be transported and stored in the vacuole, upon stress or cellular damage these are released from the vacuole and are transformed to their active aglucons by glucosidases which are located in different cell organelles (Bowles *et al.*, 2006; Morant *et al.*, 2008; Pastor *et al.*, 2013).

As previously mentioned in section 2.3.3, the accumulation of inactive (glycosylated) compounds is one of the possible mechanisms for defense priming. Our data are in line with this mechanism as we observed a large increase in compounds upon exposure to Z-3-HAC, which shared several fragment ions with D-glucose (Figure 5-11, Table 5-4), indicating that Z-3-HAC induced the accumulation of glycosylated compounds.

The presence of hexenyl glycosides in plants has already been described in literature and has primarily been reported to act as precursors for Z-3-HOL production (Jae-Hak *et al.*, 1996; Nishikitani *et al.*, 1999; Sugimoto *et al.*, 2015). Recently, it has been shown that one of the early responses (60 min) of *Arabidopsis* following Z-3-HOL exposure involves the upregulation of a putative glucosyltransferases and UDP-glucosyde hydrogenase, which may result in changes in glycosylated compounds (Engelberth *et al.*, 2013). This was confirmed in a later study by Sugimoto *et al.* (2014), in which they illustrated for 24 different plant species, including *Arabidopsis*, and *T. aestivum*, that hexenyl glucosides and hexenyl diglucosides increased after exposure to Z-3-HOL, which have antinutritional effects on *S. litura* caterpillars. It remains unknown via which mechanisms BVOCs are perceived and its signal is transduced through the cell (section 3.5)(Heil, 2014). One of the proposed mechanisms is passive diffusion across the cuticle and cell wall because of the lipophilic character of GLVs. However, Widhalm *et al.* (2015), showed that for BVOC release, passive diffusion alone does not account for observed emission rates, as this would entail such high BVOC concentrations in the cell membrane, that it would compromise its functionality and stability. They propose that other modes of transport such as lipid transfer proteins, vesicle trafficking or VOC transporters in the membrane are also needed for BVOC release. It should be investigated whether for the uptake of Z-3-HAC such active transport mechanisms play a role or whether passive diffusion alone suffices.

Recently, the enzyme responsible to form Z-3-hexenyl glucoside has been identified. Ohgami *et al.* (2015) showed *in vitro* that AtUGT85A3 produced hexenyl-glc with UDP-glucose as a sugar donor and Z-3-hexenol as a sugar acceptor. Furthermore, they showed that homologues of the UGT85 family in several other plant species also formed hexenyl-glycosides. CsGT1, the AtUGT85A3 homologue in *C. sinensis* was shown to have broad substrate specificity, including benzyl alcohol, linalool, geraniol, and Z-3-hexenol. However, GTs preferentially bind to hydroxyl groups, implying that Z-3-HAC most likely will be reduced to Z-3-HOL by an esterase before it can be glycosylated (Koichi Sugimoto, personal communication, February 25, 2017). Further research is mandatory to identify the

glycosylated compounds to unravel whether these are involved in plant defense and whether these are associated within the same pathway.

5.5.2 Z-3-HAC interferes with benzoxazinoid production

Our data revealed that Z-3-HAC treatment resulted in a downregulation of the production of the benzoxazinoid DIMBOA (Figure 5-5). Furthermore, seedlings which had been primed with Z-3-HAC and subsequently inoculated with *F. graminearum* exhibited an even lower level of DIMBOA (Figure 5-6). To our knowledge, this is the first time that the effect of a GLV on benzoxazinoid biosynthesis is shown.

Benzoxazinoids are a class of phytoalexins produced by members of the *Poaceae* family (Kokubo *et al.*, 2016). They function as defensive compounds in plants and exert negative effects against insects (Ahmad *et al.*, 2011; Wouters *et al.*, 2016a; Wouters *et al.*, 2016b), and pathogens (Morrissey & Osbourn, 1999; Martyniuk *et al.*, 2006; Ahmad *et al.*, 2011). Besides exerting direct effects, Maag *et al.* (2015) suggested that DIMBOA-Glc also acts as a defense signaling compound by signaling for increased callose deposition in sieve elements in the defense of maize against aphids as has been reported by Meihls *et al.* (2013).

Benzoxazinoids have also been implicated in the defense against FHB. While a high concentration of DIMBOA did not offer an advantage in the defense against FHB, there was a high negative correlation between DIMBOA and DIMBOA-Glc, and DIMBOA-Glc correlated with FHB resistance (Søltoft *et al.*, 2008). This was corroborated by Kettle *et al.* (2015) which demonstrated that *Fusarium* spp. which are able to detoxify benzoxazinoids are more virulent. Remarkably, it has been described that benzoxazinoids also interfere with the secondary metabolite production of *Fg*. While DIMBOA does not have any effect on the growth of the fungus, DIMBOA and DIMBOA-Glc have been shown to abolish the trichothecene production (Etzerodt *et al.*, 2015; Etzerodt *et al.*, 2016). They revealed that DIMBOA inhibited both *Tri6*, a major transcriptional regulator of trichothecene biosynthesis, and *Tri5*, encoding the first enzymatic step of trichothecene biosynthesis. However, in a strain with the nivalenol chemotype, DIMBOA did not influence trichothecene production, showing that the effect of DIMBOA on trichothecene production is dependent on the genetic background of the *Fusarium* species (Etzerodt *et al.*, 2015). It is possible that the high DON concentration in our assay can be associated with the lower DIMBOA levels in the Z-3-HAC-treated seedlings (Figure 4-12).

5.5.3 Z-3-HAC induces SA accumulation but has no effect on JA production

Based on the hemi-biotrophic lifestyle of *F. graminearum* (Figure 4-8) and RT-qPCR data (Figure 4-11), we hypothesized in Chapter 4 that Z-3-HAC enhances defense by augmenting JA-dependent responses and inhibiting SA dependent responses. Surprisingly, our phytohormone analysis revealed a strong increase in SA after Z-3-HAC treatment at 1 hai, whereas JA production is not induced by Z-3-HAC (Figure 5-8). However, because of the lack of RT-qPCR data at the investigated time points in this Chapter it remains possible that we missed possible changes in the gene expression of SA and JA biosynthesis genes and SA- and JA-dependent defense genes at these time points (Figure 5-2).

The absence of response of JA levels after Z-3-HAC exposure is rather surprising as GLVs have been shown in other plant species to induce JA accumulation. In maize, JA already reaches maximum values between 30 en 50 min after exposure to a mixture of GLVs (Engelberth *et al.*, 2004). However, JA values in aforementioned study again normalized to control levels after 180 min. To our knowledge, no studies on JA levels after GLV exposure exist in wheat, so we have no additional knowledge whether we possibly missed the JA accumulation. In a recent paper of Caarls *et al.* (2017) they report the identification of JASMONATE-INDUCED OXYGENASES (JOXs) which are induced by MeJA between 1 and 6 hours after exposure and oxygenate JA to its inactive form. It would be interesting to investigate whether JA levels indeed are quickly produced upon Z-3-HAC exposure, but are quickly transformed to its inactive form due to the absence of a MAMP/HAMP.

Recently, a transcriptomics study by Mirabella *et al.* (2015) investigated the response of *Arabidopsis* after exposure to E-2-HAL and reported that the differentially expressed genes showed a 49% overlap with the gene expression after exposure to SA, whereas there was only a 13% overlap after JA treatment. This already suggests that E-2-HAL might activate SA dependent responses and our results also disclosed a link between Z-3-HAC exposure and SA production (Figure 5-8), corroborating their findings. However, this does not entail that GLV signaling is solely SA dependent as Mirabella *et al.* (2015) found that 32% of the differentially regulated genes was unique for E-2-HAL treatment. Additionally, it cannot be ruled out that Z-3-HAC influences SA- and JA dependent genes without influencing JA biosynthesis. It should be noted that seedlings were exposed to Z-3-HAC 15 hours prior to *Fg* inoculation. Hence, at 1 hai, metabolite analysis of Z-3-HAC treated plants were actually 16 hours after Z-3-HAC treatment. The timing issue is also apparent from the gene expression analysis (Figure 4-10), compared to the phytohormone analysis (Figure 5-7). As can be seen from Figure 4-9. MeSA treatment induces the expression of PR1. Together with

our phytohormone results (Figure 5-7) one would expect that PR1 is also induced upon Z-3-HAC exposure. In Figure 4-11, no significant upregulation or PR1 can be seen in the Z-3-HAC treatment, compared to the control treatment (1.94-fold vs. 1.00-fold, respectively). However, results from Figure 4-9, are taken at 24 hours after treatment with MeSA (i.e. 40 hours after treatment with Z-3-HAC), so it remains possible that we have missed the upregulation (Figure 4-4). Thus, a holistic transcriptomics approach at these early time points is necessary to disclose whether the early increase in SA coincides with an early increase in SA biosynthesis and concomitant defense gene expression and whether Z-3-HAC influences defense genes downstream of SA and JA production.

The reduction in L-Phe levels may be attributed to an increased flux away from L-Phe, or to substrate limitation for L-Phe biosynthesis. Interestingly, in plants L-Phe is produced from chorismate (Gamborg & Neish, 1959; Tzin & Galili, 2010), which also serves as the main substrate for SA biosynthesis (Chen *et al.*, 2009). Phe is metabolized to cinnamate through action of PAL. This further branches off in other pathways, one leading to the formation of SA, and another to p-coumaric acid. The latter can be further metabolized to form flavonoids, coumarins, and lignin, amongst others (Whetten & Sederoff, 1995; Winkel-Shirley, 2001). More detailed time series of metabolites of the phenylpropanoid and shikimate pathways are warranted to investigate whether the response of Z-3-HAC on L-Phe can be attributed to substrate limitation or increased flux towards its downstream products.

We also looked into the effect of Z-3-HAC and *F. graminearum* inoculation on the hormone IAA. The IAA levels were only higher in seedlings which had been inoculated with *F. graminearum*. Remarkably, Z-3-HAC primed seedlings inoculated with *F. graminearum* showed the highest concentration (Figure 5-8). Recently, it has been shown that IAA can be produced by *F. graminearum* (Luo *et al.*, 2016). We confirmed the production of IAA by *F. graminearum* after incubation in liquid Czapek-Dox liquid medium supplemented with L-tryptophan (data not shown). However, in our inoculation experiment it remains unclear whether IAA originates from plant or from fungal biosynthesis.

In rice, IAA has already been shown to act as a virulence factor for the pathogens *Magnaporthe oryzae* and *Xanthomonas oryzae pv. oryzae* (Ding *et al.*, 2008; Wang & Fu, 2011; De Vleesschauwer *et al.*, 2014). It has been hypothesized that IAA biosynthesis induces expression of expansins which loosen the cell wall facilitating fungal penetration in the plant cells. The role of IAA in the infection process of *F. graminearum* is still ambiguous. Buhrow *et al.* (2016) investigated the effect of exogenously applied phytohormones on the severity of FHB. They found no increase in IAA levels 14 days after infection with *F. graminearum* nor an effect of exogenously applied IAA on FHB severity on DON accumulation in both susceptible and resistant cultivars. In contrast, Luo *et al.* (2016) found a

negative effect of IAA and its intermediates on hyphal growth, conidia germination and 15-ADON production in *F. graminearum*. For *F. culmorum*, another member of the *Fusarium* species complex, exogenous IAA application resulted in reduced FHB symptoms in barley, but had no effect on *in vitro* fungal growth (Petti *et al.*, 2012).

As became apparent in our detached leaf assay, in which we exposed the seedlings at different time points to MeJA, the timing of application and subsequent defense activation is of critical importance as differently timed exposures can yield different outcomes. This phenomenon may also apply to the role of IAA signaling in the pathogenicity of *F. graminearum* and clarify results from literature. A deeper understanding of the origin of the enhanced IAA biosynthesis may shed light on the question whether IAA is used as a plant defense mechanism, as was already shown in Llorente *et al.* (2008), or exploited as a virulence factor by the fungus.

5.5.4 Priming with Z-3-HAC results in a decrease of glutamate metabolism

Our targeted metabolomics studies revealed a depletion of compounds involved in glutamate metabolism after treatment with Z-3-HAC at 1 hai and 6 hai, of which the effect was greatest after inoculation with *F. graminearum* (Figure 5-9). This suggests that glutamate is metabolized to further downstream metabolites or that N under the form of glutamine is remobilized away from infected tissue.

Glutamate plays a central role in C and N metabolism of plants. Namely, the α-amino group of glutamate serves a donor for transaminases to form other amino acids and glutamate is crucial for N metabolism and translocation through the GS/GOGAT cycle is. Through action of glutamine synthetase (**GS**), NH_4^+ can bind with glutamate, forming glutamine. Together with asparagine, glutamine is an important intermediate for N transport to other tissue where it is again converted by glutamine:2-oxoglutarate aminotransferase (**GOGAT**), producing two molecules of glutamate (Forde & Lea, 2007).

In the review of Seifi *et al.* (2013b) two plant defense strategies are discussed which were coined "*endurance*" and "*evasion*". The endurance strategy aims at maintaining cell viability by transporting N under the form of glutamine and asparagine towards the infected tissue and by converting glutamate to GABA to replenish the TCA cycle. This strategy is especially useful in the defense against necrotrophs, which benefit from killing host cells whereas the evasion strategy is more efficient against biotrophs. During evasion, N will be transported away from the infected tissue, inducing PCD, which will prevent biotrophs from gaining a foothold to colonize the plant tissue. The involvement of N translocation by an upregulation of GS and a decrease in glutamate have frequently been reported after infection (Perez-Garcia

et al., 1995; Pageau *et al.*, 2006; Seifi *et al.*, 2013a; Van Bockhaven *et al.*, 2015b), which points to N transport away from the infected tissue. In our experiment, we observed a decline in glutamine levels in the Z-3-HAC + *Fg* treatment at 1 and 6 hai, while glutamate levels were lower at 6 hai, suggesting that N is translocated away from the inoculated tissue during the early phases of infection. Remarkably, glutamine levels of Z-3-HAC treated plants which were not inoculated with *F. graminearum* were not significantly different from the control treatment, showing that recognition of the fungal colonization in combination with pre-exposure to Z-3-HAC is necessary to induce N transport. In addition, inoculation with *F. graminearum* as single treatment does not affect glutamate or glutamine levels. Furthermore, the higher drop in glutamine and glutamate levels might induce PCD faster in primed cells, impeding the infection of *F. graminearum* during its biotrophic phase.

It would be interesting to observe glutamate and glutamine levels in neighboring healthy tissue. However, in this study, we inoculated the complete leaf sheath with a conidia suspension of *F. graminearum* and during sampling, only the leaf sheath was excised for further analyses. So, we were not able to investigate whether the decrease of glutamine in the infected leaf sheaths coincides with increased N levels in healthy tissue. These results should also be nuanced as we measured the net effect of both plant and fungal N metabolism. Fungal pathogens are dependent on the N metabolism of the host to grow and preferentially uses glutamate and glutamine as primary N sources (Bolton & Thomma, 2008).

Z-3-HAC had a strong reducing effect on L-arginine levels, which were significantly lower at 1 and 6 hai, and had the highest effect in Z-3-HAC+*Fg* seedlings. This may point to an inhibiting effect on L-arginine biosynthesis, substrate limitation for L-arginine production, or to an increase of the metabolism of L-arginine, resulting in higher levels of nitric oxide and polyamines (**PAs**), which would deplete L-arginine levels. However, in our samples the levels of the PA's putrescine and spermidine were below the limit of detection. Furthermore, spermine could not be detected using our method. Based on this evidence, a higher production of PA's seems unlikely to account for the low levels of L-arginine.

GABA is at the interface of the N and C metabolism. Through action of glutamate decarboxylase, glutamate is converted to GABA which can be fed into the TCA cycle, thereby bypassing two steps of the cycle. We observed only a significant effect at 6 hai, where Z-3-HAC+*Fg* seedlings exhibited the lowest levels of GABA. On other time points, no effects could be seen. Besides maintaining cell viability by replenishing the TCA cycle, GABA has also been implicated in signaling and PCD (Loughrin *et al.*, 1994; Seifi *et al.*, 2013b). An intriguing example of the involvement of GABA in GLV signaling is the study of Mirabella *et al.* (2008). They observed that E-2-HAL inhibited root growth of *Arabidopsis*, this response was however lost in *her1* mutant plants, which encodes a γ-amino butyric acid transaminase (GABA-TP) and accumulated high levels of GABA. They were able to rescue the root

inhibition in the WT plants by applying high levels of GABA. However, only compounds with an α,β-unsaturated carbonyl group, such as E-2-HAL were able to inhibit root growth whereas compounds such as E-2-HOL and Z-3-HOL did not have any effect on root growth (Mirabella *et al.*, 2008). As Z-3-HAC does not contain such a group, it remains to be determined whether GABA is also involved in Z-3-HAC signaling, or that the decrease in GABA can be attributed to substrate limitation of glutamate.

5.6 Conclusion

This chapter aimed at further uncovering underlying priming mechanisms and represents the first study that investigated metabolome changes after priming by GLVs in the context of plant-pathogen interactions. Earlier time points were included in this chapter. It became apparent that results from Chapter 4 could not always be linked to results from this chapter. This illustrates that including smaller and earlier time frames are necessary to investigate plant-pathogen interactions and dynamics (Figure 5-2).

Using an untargeted metabolomics approach, we were able to identify compounds which increased after exposure to Z-3-HAC. Based on *in silico* analyses and tandem MS we found strong evidence that these compounds contain glycoside groups, which suggests that Z-3-HAC activates glycosylation processes in wheat, which may contribute to the priming response. We putatively identified the presence of HexVic, a diglycoside of hexenyl in Z-3-HAC treated plants. This indicates that Z-3-HAC is metabolized in the cells and might act as signaling compound in plant defense. Our metabolomics approach also revealed that Z-3-HAC interferes with benzoxazinoid biosynthesis, resulting in a downregulation of DIMBOA. However, more research is needed to elucidate whether Z-3-HAC might prime for enhanced production of other benzoxazinoids, offering increased defense.

In this study, we also employed a targeted approach to investigate some key metabolites involved in plant defense. Surprisingly, we found a large increase in SA in Z-3-HAC treated leaves, whereas JA biosynthesis was not influenced, which indicates that Z-3-HAC induces processes upstream of SA production. Finally, we found for several metabolites involved in glutamate metabolism a decrease in Z-3-HAC + *Fg* treated seedlings. While more information is still needed, this lays the foundation of future research which investigates whether Z-3-HAC activates responses aimed at translocating N away from the infected tissue.

5.7 Appendix

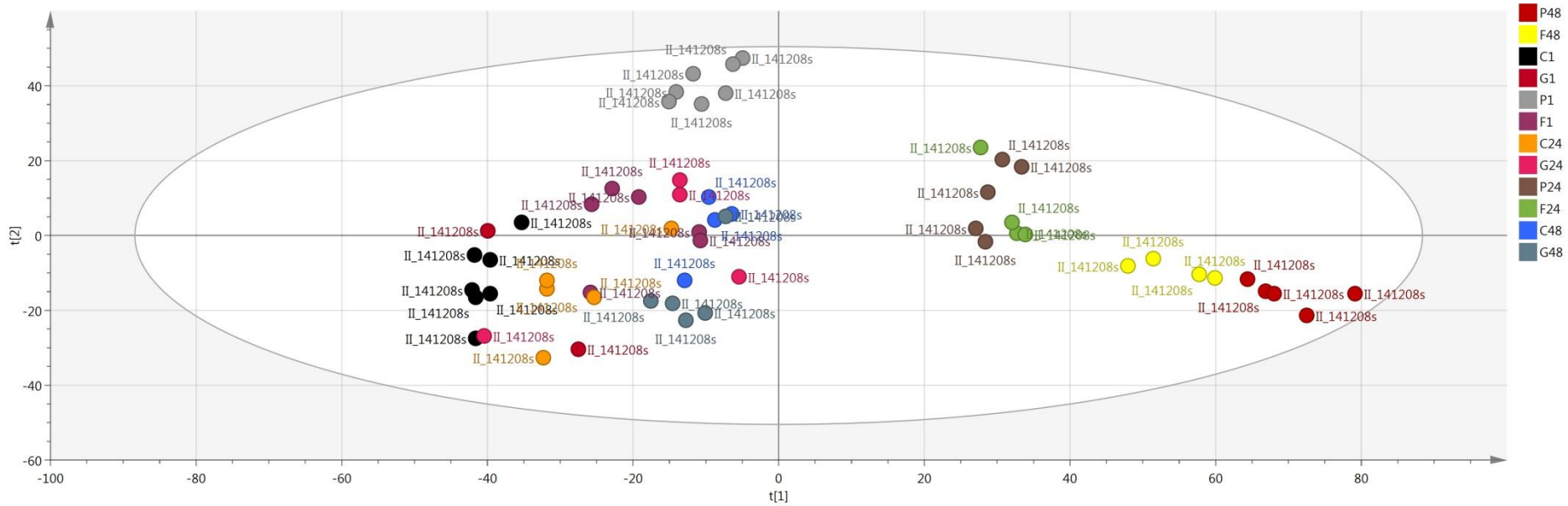


Figure 5-10: Principal Component Analysis score plots of the full scan m/z data for the different time points. Circles in the PCA plot represent biologically independent samples. Circles sharing the same color received the same treatment. Seedlings were placed in bags and a mock treatment or Z-3-HAC was applied on a piece of filter paper according to the experiment. A continuous air flow (600 ml min^{-1}) was supplied to the cuvette to prevent an increase of the relative humidity. Seedlings were kept inside the cuvettes overnight and the day after, seedlings were taken from the cuvettes. Leaf sheaths were subsequently challenged with a mock treatment or a conidia suspension of *F. graminearum.* The following days, leaf sheaths were sampled and prepared for HPLC - MS/MS analysis. Treatment abbreviations: C, control; G, green leaf volatile (Z-3-HAC) treatment; P, primed (Z-3-HAC+F. graminearum) treatment); F, *Fusarium graminearum* treatment. Numbers behind the treatments in the legend represent the time points after inoculation. The ellipse depicts the Hotteling T2 95% confidence interval.

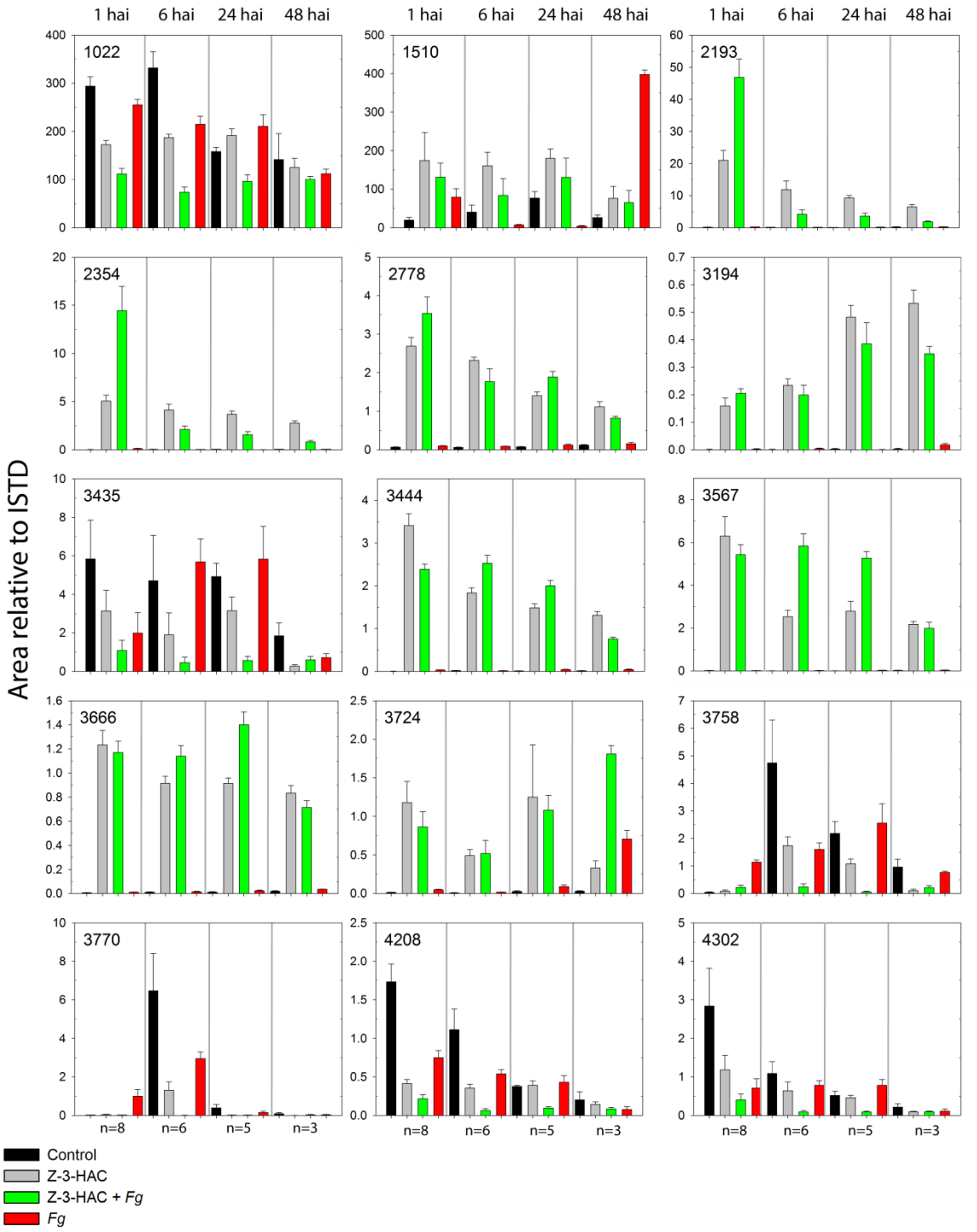


Figure 5-11: Peak areas of the metabolites from Table 5-3 for the different treatments and time points. Based on the VIP scores, loading factors and S-plots of the models, the metabolites which contribute the most to the predictability of the model were selected. Peak area relative to the internal standard (ISTD) is shown. As internal standard, a deuterium labelled analytical standard of 100 pg µl-1 d6-abscicic acid (OlChemIm, Olomouc, Czech Republic) was used. Each biological replicate consists of 100 mg fresh weight of pooled leaf sheaths. Error bars represent SE.

Table 5-4: Fragment ions of metabolites which were most abundant in the Z-3-HAC and Z-3-HAC + *Fg* treatment share fragment ions of D-glucose. The 20 fragments with the highest abundance are shown. Fragment ions which are shared between metabolites and D-glucose are depicted in bold and underlined. Fragment ions which are shared with other metabolites are depicted in bold.

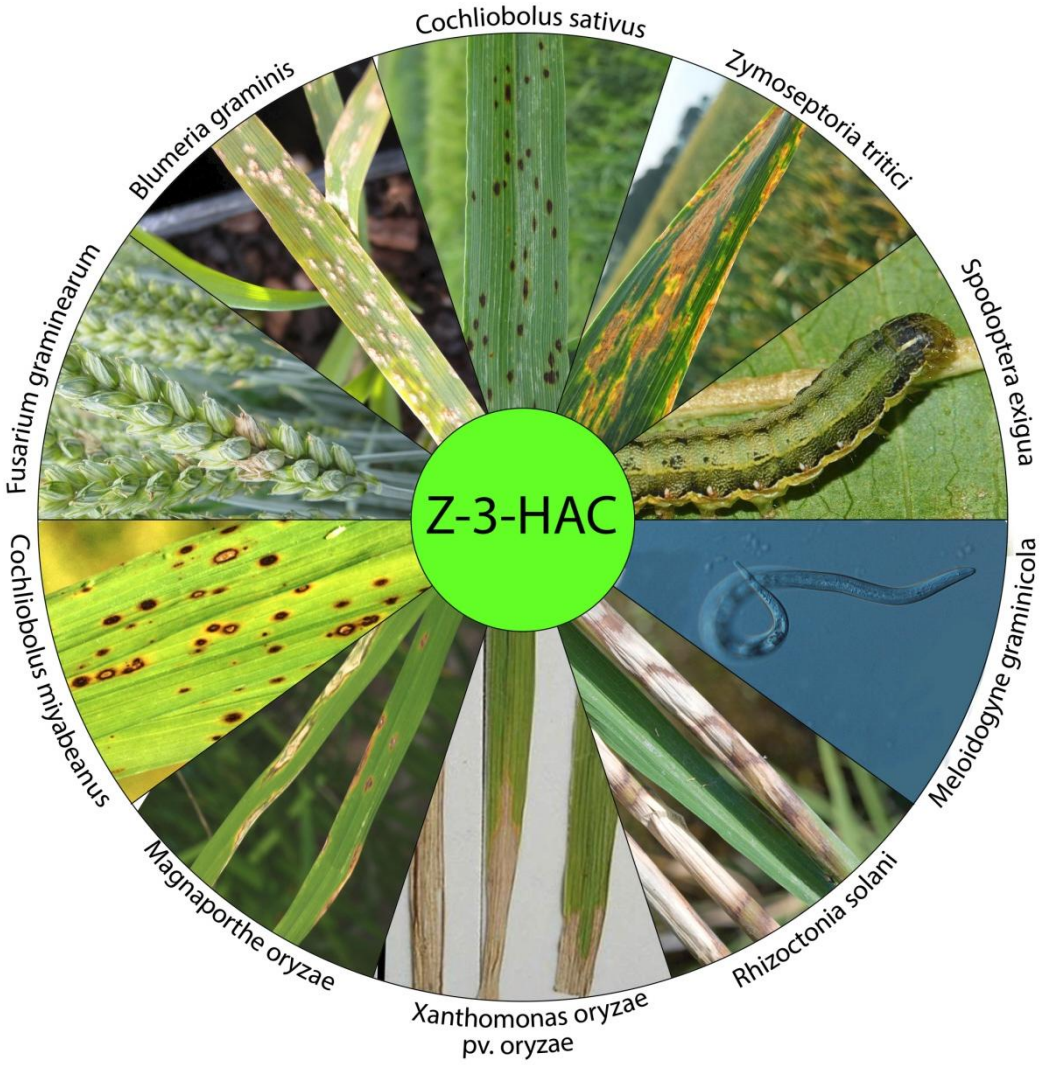
Metabolite											
D-glucose		**1510**		**2193**		**2354**		**2778**		**3194**	
m/z	relative intensity	m/z	relative intensity	m/z	relative intensity	m/z	relative intensity	m/z	relative intensity	m/z	relative intensity
57.03420	4.47	**53.03934**	1.15	70.06579	12.67	69.03423	31.58	55.05507	35.82	60.08158	57.97
61.02915	11.34	**55.01856**	0.97	72.08142	11.59	70.06581	25.5	**57.03429**	32.18	**85.02898**	9.48
69.03415	16.08	55.05501	4.77	**85.0289**	15.14	**81.03404**	23.94	**69.03419**	61.9	**89.0602**	12.43
71.04955	1.03	**57.03426**	4.08	86.09692	25.96	**85.02897**	76.95	71.0498	67.74	107.0495	38.02
73.02902	1.6	**61.02915**	8.2	**89.06017**	100	86.09696	98.08	**81.03403**	43.03	120.0444	10.12
81.03396	5.78	**69.03416**	13.88	90.06352	6.67	**97.02874**	46.04	81.07043	39.03	121.0285	100
85.0886	100	71.04977	2.45	**97.02871**	4.36	109.0287	37.6	83.04968	47.12	**121.065**	34.82
87.0448	4.05	73.029	3.02	102.0915	4.47	**116.0707**	43.04	83.08605	44.44	136.0756	23.53
91.03954	6.42	73.06543	35.21	**116.0708**	26.97	123.0436	22.8	**85.02891**	79.59	139.0728	10.33
97.02874	17.46	81.0341	3.79	**121.065**	8.01	137.0596	100	93.07031	30.71	147.0438	14.94
99.04425	5.02	**85.0289**	100	133.0859	33.67	149.0596	25.07	95.08595	41.35	162.0548	63.52
109.02869	8.83	86.03223	2.27	143.1178	13.33	**151.0753**	53.04	111.0806	100	164.0342	36.37
127.03885	21.5	**87.04455**	2.3	157.1336	12.44	161.0594	30.37	123.0805	36.57	177.0547	9.44
131.95009	1.51	**91.03941**	4.21	177.1125	4.74	165.0544	24.29	**127.039**	33.13	208.0604	18.07
132.9850	1.5	**97.02876**	21.3	187.0749	5.32	173.1281	30.63	135.0804	35.93	222.0758	18.87
144.06502	1.32	**99.04454**	4.43	254.1749	6.31	175.0754	30.8	139.0753	97.72	**301.1254**	28.45
144.9592	1.11	**109.0286**	11.11	300.18	13.96	179.0703	57.21	141.0909	89.84	343.136	54.12
145.04947	19.91	115.0392	1.17	300.2164	4.59	203.0702	63.95	**151.0753**	51.76	344.1393	22.95
163.06020	1.35	**127.039**	18.45	**301.125**	49.41	245.0797	25.37	209.1169	54.47	387.1252	17.44
180.08672	3.43	**145.0493**	8.21	301.1828	5.05	317.0831	28.62	295.1171	37.48	389.177	14.85

Table 5-4 Continued

Metabolite									
D-glucose		**3444**		**3567**		**3666**		**3724**	
m/z	relative intensity	m/z	relative intensity	m/z	relative intensity	m/z	relative intensity	m/z	relative intensity
57.03420	4.47	73.02893	18.01	**55.05511**	1.48	**55.05493**	7.29	**69.03424**	12.98
61.02915	11.34	**85.02895**	24.35	61.02913	1.6	**69.03414**	29.36	70.06585	33.5
69.03415	16.08	**97.02888**	35.4	**69.03416**	7.91	72.08138	11.12	73.02911	11.3
71.04955	1.03	115.0389	44.85	71.0498	2.02	73.02904	43.14	**85.02898**	49.66
73.02902	1.6	**127.0398**	9.87	73.02909	4.02	73.04736	10.48	**97.02882**	15.31
81.03396	5.78	**145.0496**	30.3	**81.03407**	4.36	**85.02876**	13.02	113.0712	14.04
85.0886	100	150.0757	11.13	**85.02892**	80.82	**87.04446**	20.8	121.0286	67.32
87.0448	4.05	151.0396	10.94	86.03217	2.18	89.06015	78.77	**127.0391**	15.23
91.03954	6.42	**163.06**	29.2	**87.04447**	1.82	91.05786	9.67	141.0182	29.79
97.02874	17.46	233.1393	9.63	89.06019	5.48	98.97556	7.04	**145.0496**	13.59
99.04425	5.02	241.0692	14.53	**97.02879**	19.3	**99.0444**	13.01	164.0705	78.69
109.02869	8.83	245.138	25.82	**99.0444**	20	103.0395	12.45	165.0738	11.93
127.03885	21.5	259.0814	14.34	103.0393	94.03	111.0445	7.14	209.0443	30.4
131.95009	1.51	263.1484	100	104.0425	2.89	**127.0387**	16.15	259.0865	29.11
132.9850	1.5	264.1512	10.41	**109.0287**	13.52	133.0858	24.3	265.0857	12.68
144.06502	1.32	280.174	8.86	**127.039**	100	158.9963	7.56	272.0944	11.39
144.9592	1.11	295.1016	93.22	128.0422	3.76	219.0176	20.84	289.1329	15.55
145.04947	19.91	412.2169	31.33	**145.0494**	27.82	237.0277	12.14	301.1332	100
163.06020	1.35	413.1285	12.29	**163.0598**	5.9	301.0953	100	302.1364	42.57
180.08672	3.43	413.2993	22.95	187.0599	4.17	319.1056	51.04	318.1596	17.01

Chapter 6 The Priming Potential of Z-3-HAC in Wheat and Rice

Ameye M, Audenaert K, Höfte M, Kyndt T, Le Mire G, Jijakli MH, Smagghe G, Haesaert G, in preparation for Molecular Plant Pathology



6.1 Abstract

GLVs have been assigned a role in plant resistance against pathogens. They can exert both direct and indirect effects against plant pathogens by inhibiting pathogen growth and enhancing plant defense, respectively. Because of these properties, GLVs may constitute a novel class of biocontrol agents in agronomy. In the previous chapter, we demonstrated that exposure to Z-3-HAC induces accumulation of SA, which may counteract other defense signaling pathways and as such induce susceptibility in other pathosystems. To elucidate whether the GLV Z-3-HAC contributes to enhanced resistance, we performed several bio-assays in different pathosystems for wheat and rice.

We showed that Z-3-HAC enhanced wheat resistance against *Fusarium* spp. and *Cochliobolus sativus*, decreased the mortality of the caterpillar *Spodoptera exigua*, and induced susceptibility to *Blumeria graminis* and *Zymoseptoria tritici*. For rice, we also found contrasting outcomes for different pathosystems. Namely, Z-3-HAC enhanced resistance against *Magnaporthe oryzae*, *Xanthomonas oryzae, pv. oryzae, Rhizoctonia. solani* and the root knot nematode *Meloidogyne graminicola,* but had no effect on resistance against *Cochliobolus miyabeanus*.

Additionally, we demonstrated that the defense responses after Z-3-HAC exposure could not completely be attributed to SA or JA dependent defenses.

Thus, Z-3-HAC may constitute a promising tool in the biocontrol against a broad range of pathogens, but because of the risk of making plants more susceptible to other pathogens, the use of other agrochemicals is indispensable in agronomy.

6.2 Introduction

In **Chapter 4** we have shown for the first time that Z-3-HAC is effective in enhancing wheat defense against *F. graminearum* infection via a priming mechanism. We found that pre-exposure to the GLV Z-3-HAC led to a lower percentage diseased spikelets and smaller lesion length. Results from the follow-up experiments revealed that the enhanced defense could be attributed to enhanced JA mediated defense, pointing to a model where priming by Z-3-HAC enhances the JA dependent defense pathway. It remains debatable whether GLVs solely activate JA signaling, which results in enhanced resistance or that GLV signaling acts via another, yet undiscovered pathway (Section 3.7). Indeed, results from Chapter 5 showed that Z-3-HAC greatly induced production of SA, which suggests that GLV may interfere with SA and JA signaling. As suggested in section 1.1, GLVs might constitute a potent biocontrol agent. However, because of known antagonistic defense signaling pathways (De Vleesschauwer *et al.*, 2014), one may speculate that activation or priming of defense pathways by GLVs might activate antagonistic defense pathways and even induce susceptibility in other pathosystems. Thus, a thorough screening of efficacy against different pathogens is necessary to elucidate whether GLVs might prove a promising agronomic tool.

To obtain a better understanding of the priming potential of Z-3-HAC in the economically important monocot crops wheat and rice (*Oryza sativa* L.) against a broad spectrum of devastating plant pathogens, we selected several pathogens, an insect herbivore and a nematode, each with varying infection strategies against which wheat or rice employ different defense strategies. For wheat, we selected the fungal pathogens *Fusarium graminearum* (Schwein), *F.* poae (Peck), *Blumeria graminis* (DC.)*, Cochliobolus* sativus (S. Ito & Kurib.), *Zymoseptoria tritici* (Fuckel), and a generalist insect herbivore, *Spodoptera exigua* (Hübner). For rice, we used *Cochliobolus miyabeanus* (S. Ito & Kurib.), *Magnaporthe oryzae* (T. T. Hebert)*, Rhizoctonia solani* (J.G. Kühn)*,* the bacterial pathogen *Xanthomonas oryzae pv. oryzae* (Uyeda & Ishiyama), hereafter abbreviated to *Xoo* and the rice root knot nematode *Meloidogyne graminicola* (Golden & Birchfield).

6.3 Materials and Methods

6.3.1 SA and JA analysis

In Chapter 5, we already determined the levels of the plant hormones SA and JA upon Z-3-HAC exposure (Figure 5-8). However, as we do not have data on the SA and JA levels in rice upon Z-3-HAC exposure, we determined this in a similar manner as previously described in Chapter 5. In short, we exposed 3 week old rice seedlings cv. Kitaake to Z-3-HAC and sampled leaves at 1, 17, 22 and 40 hours after exposure. Phytohormone levels were

determined using the methodology as previously described in section 5.3.1 and 5.3.2, and using the accurate mass and retention time reported in Table 5-1. In previous experiments (data not shown) we have determined that there is a linear relationship between the peak area relative to the internal standard and the mass of phytohormone present in the sample.

6.3.2 Pathogens

For wheat, the following pathogens were used: a GFP transformant of *Fusarium graminearum* strain 8/1 (Jansen *et al.*, 2005) (kindly provided by dr. Karl Heinz-Kogel), a field isolate of *F. poae* strain 2516 (Vanheule *et al.*, 2016), a field isolate of *Blumeria graminis, Cochliobolus sativus* strain MUCL 46854, and *Zymoseptoria tritici* strain TO1187.

The following strains of pathogens of rice were used in this study: *Cochliobolus miyabeanus* strain Cm988 (De Vleesschauwer *et al.*, 2010), *Xoo* strain PXO99 (Xu *et al.*, 2013), *Magnaporthe oryzae* strain VT5M1 and *Rhizoctonia solani* anastomosis group AG1-1A, both kindly provided by dr. Monica Höfte.

6.3.3 Inoculum preparation

F. graminearum and *F. poae* isolates were grown on potato dextrose agar (PDA) for seven to ten days at 20°C under a regime of 12 h dark and 12 h combined UVC and UVA light (2x TUV 8W T5 and 1x TL 8W BLB, Philips, the Netherlands). *C. miyabeanus* and *M. oryzae* were grown on PDA and complete medium, respectively, for 7 days at 28°C in the dark and placed for two days under a regime of 12 h dark and 12 h combined UVC and UVA light (2x TUV 8W T5 and 1x TL 8W BLB, Philips, the Netherlands) to induce sporulation. Conidia were harvested by adding a solution of 0.01% Tween80 to the PDA plates and rubbing the mycelium with a drigalski spatula. Subsequently, the *F. graminearum* and *F. poae* suspensions were diluted to a final concentration of 5×10^5 conidia mL^{-1}. The *C. miyabeanus* and *M. oryzae* suspension was diluted in 0.5% gelatine (type B from bovine skin; Sigma-Aldrich G-6650) to a final concentration of 2.5×10^4 conidia mL^{-1}.

Xoo strain Px099 was grown on Sucrose Peptone Agar (SPA) medium at 28°C for 5 days. Subsequently, plates were scraped off with an L-shaped spreader and diluted in distilled water to an OD_{620} of 0.5.

A field isolate of *Blumeria graminis* was used in this study. Cultures were maintained at 15°C with a photoperiod of 16 h on detached leaves of the susceptible wheat cv. Cerco which were placed on a Petri dish containing water agar (7.5 g L^{-1}) amended with benzimidazole (40 mg L^{-1}) (Troch *et al.*, 2014).

Zymoseptoria tritici strain TO1187 was grown on PDA. After plates were fully grown, conidia were harvested and diluted to a final concentration of 1×10^6 conidia mL^{-1} for the inoculation.

R. solani was grown on PDA. Sterilized toothpicks were placed on PDA plates with an agar plug with *R. solani* in the center. Plates were incubated at 28°C in the dark for three days to allow *R. solani* to colonize the toothpicks, which were later used in the infection assay.

6.3.4 Plant material

Seeds of wheat (*Triticum aestivum* L.) var. Sahara were germinated and grown in pots (8.5 cm diameter x 6.5 cm height) in commercial potting soil (Structural type 1; Snebbout n.v., Kaprijke, Belgium) in a growth chamber (18°C, 16 h-8 h, light-dark regime) for two weeks. Rice (*Oryza sativa* L.) plants used in this study included the *japonica* var. *Kitaake, Bomba* and *indica* var. CO39. Seeds were surface sterilized with 2% sodium hypochlorite solution for 2 min, rinsed three times in sterile distilled water, and germinated in the dark on wet sterile filter paper at 28°C. Five days later, germinated seeds were transferred to plastic containers with vermiculite containing modified Hoagland solution and placed in a growth chamber (28°C, 16 h-8 h, light-dark regime). One week later, the seedlings were transferred to commercial potting soil (Structural type 1; Snebbout n.v., Kaprijke, Belgium). Plants were watered daily and fertilized weekly with 1 g L^{-1} $(NH_4)_2SO_4$ and 2 g L^{-1} $FeSO_4 \cdot 7H_2O$.

6.3.5 Experimental design

6.3.6 Inoculation assays

All assays were performed at least twice in time. An independent biological replicate represents a different individual seedling.

6.3.6.1 Fusarium spp. and *Cochliobolus sativus*

To investigate whether pre-exposure of wheat seedlings to Z-3-HAC (Sigma-Aldrich) leads to enhanced defense against a subsequent infection by *F. graminearium, F. poae* or *C. sativus*, we performed a detached leaf assay experiment following the procedure described in **Section 4.3.6.** In short, 4 cm leaf segments were cut from the tip of the leaves of two-week old seedlings (n=10 and n=18 for *Fusarium spp*. and *C. sativus*, respectively). These leaves were placed on their abaxial surface in Petri dishes containing 1.5% bacteriological agar amended with 40 mg L^{-1} benzimidazole (Sigma-Aldrich). The centre of the leaf segment was wounded using a sterile inoculation needle after which a droplet of conidia suspension of 5 x 10^5 conidia mL^{-1} (*Fusarium* spp.) or 2.5 x 10^4 conidia ml^{-1} (*C. sativus*) was placed on the wound. Lesion length was measured for *Fusarium* spp. the following days using Cell^F (Olympus Europe, Hamburg, Germany). Lesion area was measured at 72 hai using APS Assess 2.0 for *C. sativus*.

6.3.6.2 Blumeria graminis

The inoculation assays of wheat with *Blumeria graminis* are adapted from Troch *et al.* (2014). In a first experiment, leaves of two-week-old wheat seedlings (cv. Sahara)(n=12) were cut off and placed on Petri dishes containing 1.5% bacteriological agar amended with 40 mg L^{-1} benzimidazole. Each Petri dish contained two leaves from a primed seedling alternating with two leaves from a control seedling and a leaf from a susceptible control (cv. Cerco). In a second experiment, we used 14 day old seedlings which were planted in small pots (n=6). For each replicate, we placed a primed, a nonprimed and a susceptible control seedling (cv. Cerco) in the inoculation tower. Inoculation for both experiments was performed using a settling tower measuring 300 mm high and 103 mm diameter for uniformly dispersing the conidia across the Petri dish (Figure 6-1). After carefully blowing the conidia in the inoculation towers, Petri dishes remained for 5 minutes inside the tower, allowing the conidia sufficient time to settle on the leaves.

Figure 6-1: Second inoculation assay of *Blumeria graminis*. Wheat seedlings were grown in small pots until they were two weeks old. Left photo: each replicate consisted of a seedling which was treated with Z-3-HAC, a control seedling which was not treated, and a susceptible control (cv. Cerco). Right photo: seedlings were subsequently placed in an inoculation tower in which spores of *B. graminis* were evenly dispersed over the seedlings.

After 6 days, infection was scored using a scale from 0 to 4, where 0 = no colonization; 1 = minute colonies with few conidia produced; 2 = colonies with moderately developed hyphae, but few conidia; 3 = colonies with well-developed hyphae and abundant conidia, but colonies not joined together; and 4 = colonies with well-developed hyphae and abundant conidia, and colonies mostly joined together (Troch *et al.*, 2014).

6.3.6.3 Zymoseptoria tritici

The susceptible wheat variety 'Avatar' was used for the greenhouse trials. The wheat plants were grown in greenhouses of the Gembloux agro-bio tech faculty. When the plants reached the 3-4 leaf stage, half of the plants were pre-treated with Z-3-HAC. For both treated and control plants, there were 9 pots of 10 plants (n=90). Plant inoculation was performed 24 hours after treatment, by spraying the plants of each pot with 30 mL of the conidia suspension (1×10^6 conidia mL^{-1}) amended with 0.05% Tween20 (Sigma-Aldrich). Immediately after inoculation, each pot was covered with a transparent polyethylene bag for three days in order to ensure high humidity to facilitate conidia germination. The disease level was scored at 28 days post-inoculation by determining the percentage of the third leaf area covered with symptomatic lesions. To analyze the results an ordinal class system was used: 0 for no symptoms; 1: 0-10% diseased leaf area; 2: 10-20% diseased area; 3: >20% diseased area.

6.3.6.4 Spodoptera exigua

The effect of Z-3-HAC treated wheat leaves on the growth and development of beet armyworm (*S. exigua*) larvae was examined, using a feeding experiment. Ten caterpillars were placed in three separate Petri dishes each of which the lid contained a hole covered by netting, providing sufficient fresh air to the caterpillars and preventing them from escaping. Each day, freshly primed and control leaves (var. Sahara) were provided to the caterpillars. The average weight and the developmental stage were monitored daily, by weighing the caterpillars and measuring the width of the head capsule, respectively (Table 6-1). Differences in survival between the two treatments was calculated using the Kaplan-Meier method (Kaplan & Meier, 1958). Because of the small size of the wheat leaves, we were not able to measure the leaf surface which was eaten.

Table 6-1: Head capsule width associated with each instar phase of *S. exigua*

Size head capsule (mm)	Larval stage
width < 0.45	First instar
0.45 < width < 0.7	Second instar
0.7 < width < 1.12	Third instar
1.12 < width < 1.8	Fourth instar
1.8 < width	Fifth instar

6.3.6.5 Cochliobolus miyabeanus

To inoculate rice leaves with *C. miyabeanus*, we followed the procedure described in Van Bockhaven *et al.* (2015a). Second youngest leaves of 10-week-old rice plants were cut and placed in a Petri dish containing water overnight, to allow the leaves to destress. The following day, Z-3-HAC treated- and control leaves were placed in Petri dishes containing wet paper towels to allow sufficient moisture, preventing desiccation of the leaves. Subsequently, the leaves were either sprayed (n=6) or drop-inoculated (n=11) with a conidia suspension (1 x 10^4 conidia ml^{-1}). At 72 hai, plant resistance was assessed by measuring the lesion area (drop inoculation) or counting the number of lesions (spray inoculation).

6.3.6.6 Magnaporthe oryzae

Inoculation of rice with *M. oryzae* was performed following the procedure described in De Vleesschauwer *et al.* (2010). Five-week-old plants (var. CO39) were misted with the conidial suspension (1 mL per plant) of *M. oryzae,* using an artist airbrush powered by an air compressor. Inoculated plants were kept in a dew chamber for 24h (+/- 92% relative humidity; 28 +/- 2 °C) to promote infection and subsequently transferred to a growth chamber for disease development. *M. oryzae* inoculated plants (n=9) were scored for disease development 6 days after inoculation, based on the number and type of lesions. A susceptible type of lesion contains a gray center, indicative of sporulation, while resistant (nonsporulating) necrotic lesions have a yellowish center. The latter type of lesions are thus indicative of pathogens which do not complete the disease cycle (Valent *et al.*, 1991).

6.3.6.7 Xanthomonas oryzae pv. oryzae.

Inoculation of rice with *Xoo* was performed following the procedure described in Xu *et al.* (2013). The second youngest leaves (n=10) (var. Kitaake) were clipped with a pair of scissors dipped in the *Xoo* suspension. Lesion length was measured after 14 days. Subsequently, 15 cm leaf segments were cut and ground in sterile white sand with 10 mL physiological water (8.5 g L^{-1} NaCl). A dilution series was made and 10 µL droplets were pipetted on sucrose peptone agar plates. After 72 hours, the colony forming units (CFU) were counted.

6.3.6.8 Rhizoctonia solani

For the inoculation of rice seedlings (n=8) with *R. solani* (var. Kitaake), we followed the procedure described in Taheri *et al.* (2007). Toothpicks colonized with hyphae of *R. solani* were placed in the leaf sheath of the main tiller (var. Kitaake) and covered with parafilm to ensure a humid environment for infection. After 72 hours, parafilm was removed and the lesions were measured.

6.3.6.9 Meloidogyne graminicola

Inoculation of roots of rice seedlings (var. Bomba) with *M. graminicola* nematodes was performed following the procedure described in Nahar *et al.* (2011). Ten primed and ten control rice seedlings of 16 days old rice plants var. Bomba were inoculated with 200 second-stage juveniles of *M. graminicola* per plant. After 15 days, infection level was evaluated using acid fuchsin staining. The number of galls per plant and developmental stage was observed to determine rice susceptibility to *M. graminicola*. To visualize the galls, roots were boiled for 3 min in 0.8% acetic acid and 0.013% acid fuchsin. They were subsequently washed and destained in acid glycerol. This experiment was repeated at two different time points.

6.3.7 Data analysis

Data were checked for normality using the Shapiro Wilk test, equality of variances was checked using Levene's test. Statistical comparisons between different treatments were calculated using Student's t-test. If ordinal scoring systems were used, the Kruskal-Wallis test was used to calculate statistical differences between two treatments. Generalized linear model (GLM) analyses with treatment and time as fixed factors were performed to calculate significance of differences between the levels of SA and JA in wheat and rice (SPSS 22; IBM).

6.4 Results

6.4.1 SA and JA levels in wheat and rice

In Chapter 5 we observed a large increase in SA levels upon Z-3-HAC exposure in wheat. We additionally performed a phytohormone analysis of rice seedlings upon Z-3-HAC exposure (Figure 6-2). For wheat, we took a subset of data previously presented in Figure 5-8, these data represent SA and JA levels after 1, 6, and 24 hours after inoculation. However, seedlings were already exposed for 15 hours to Z-3-HAC and thus represent values of 16, 21 and 39 hours after Z-3-HAC exposure. Treatment of wheat seedlings resulted in a significant increase in SA levels, whereas JA levels were not influenced (Figure 6-2). On the other hand for rice, we did not observe a significant effect of Z-3-HAC exposure on SA levels, whereas JA levels were significantly higher in Z-3-HAC treated seedlings (Figure 6-2).

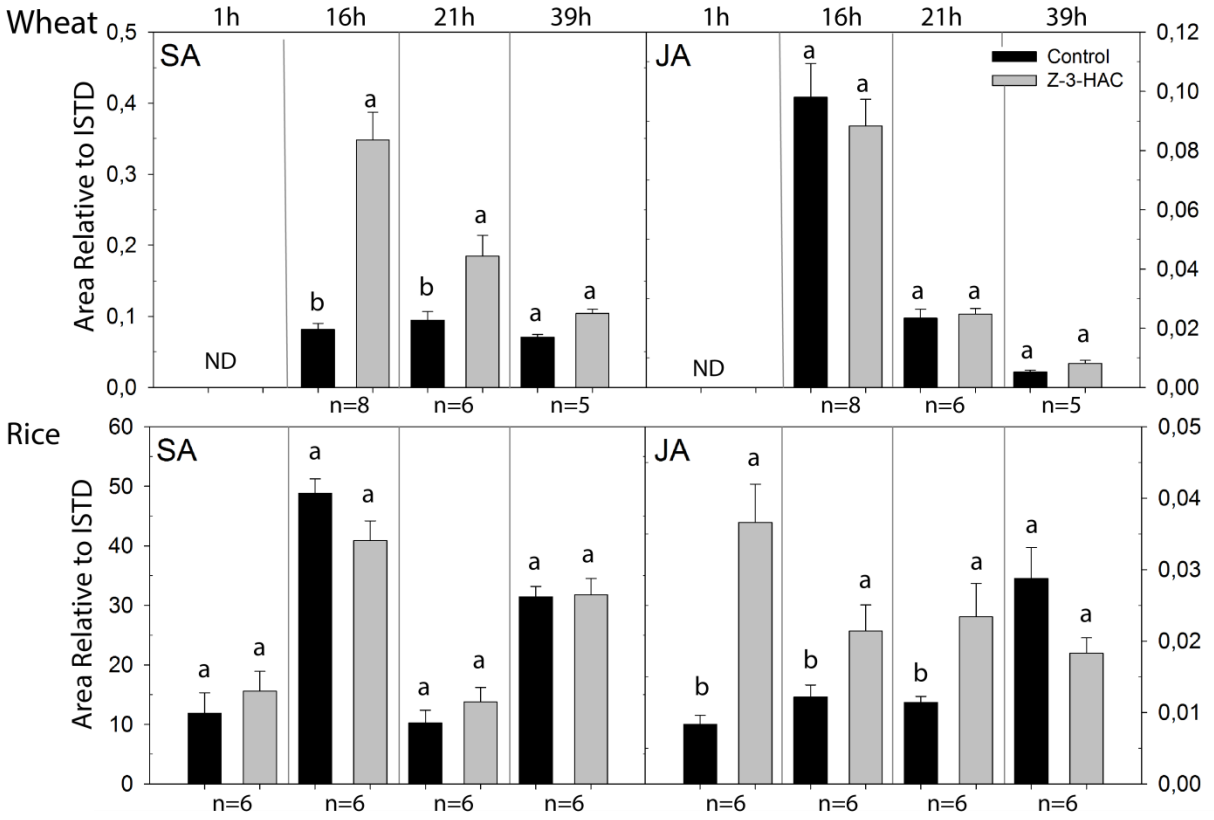


Figure 6-2: Salicylate (SA) and jasmonate (JA) analysis (area relative to the internal standard) of wheat (upper graph) and rice (bottom graph) seedlings after exposure to Z-3-HAC. Values for the wheat seedlings represent a subset of data previously presented in **Figure 5-8**. **Peak area relative to the internal standard (ISTD) is shown. As internal standard, a deuterium labelled analytical standard of 100 pg µl^{-1} d_6-abscicic acid (OlChemIm, Olomouc, Czech Republic) was used.** The number of biological replicates per time point per treatment bar are shown beneath the bars. Error bars represent SE. Significance of differences was calculated by performing a generalized linear model (GLM) procedure (α=0.05). Abbreviations: ISTD, internal standard; ND: not determined.

6.4.2 Wheat

6.4.2.1 Fusarium spp.

To test whether Z-3-HAC can increase resistance against two species of *Fusarium*, we pre-treated wheat seedlings with Z-3-HAC and inoculated them with a conidia suspension of *F. graminearum* (n=10) or *F. poae* (n=10). For both species, Z-3-HAC treatment significantly reduced lesion length with 22% (P<0.05) and 36% (P<0.05) at 72 hai for *F. graminearum* (Figure 6-3A) and *F. poae* (Figure 6-3B), respectively. For *F. poae*, we did not observe a clear progression of the infection front in the Z-3-HAC treated leaves, indicating that *F. poae* was not able to colonize these leaves beyond the wounds that were made (Figure 6-3B).

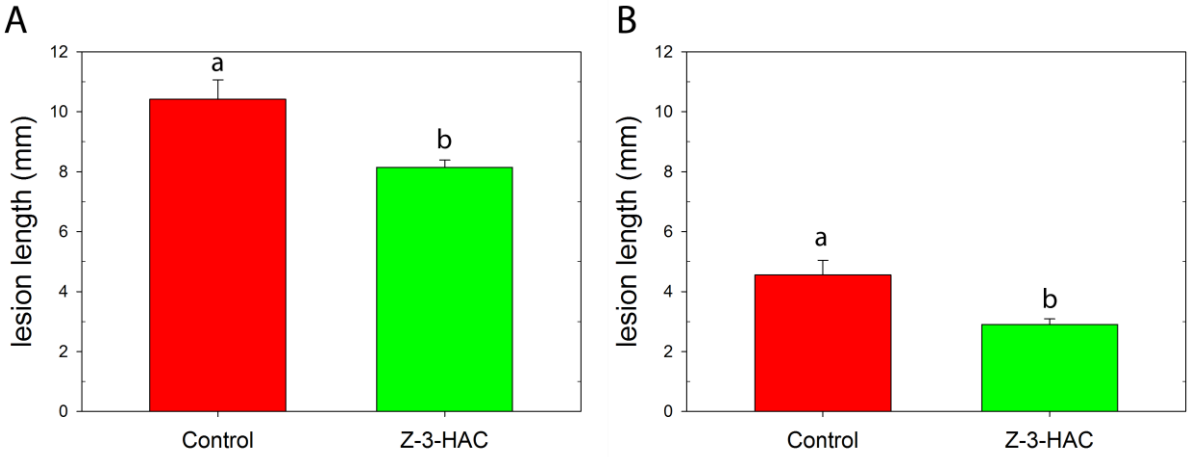


Figure 6-3: Leaves of seedlings pre-exposed to Z-3-HAC show smaller lesions compared with the control treatment at 72 hai. Leaves were cut from the seedlings and subsequently wounded, after which a droplet of a conidia suspension of *F. graminearum* (A) or *F. poae* (B) (5 x 10^5 conidia mL^{-1}) was applied on the wound. Lesion length was monitored at 72 hai. Bars represent means of 10 biological replicates. The experiment was done twice independently in time. Data represent the results of one experiment. Bars depicted with different letters indicate significant differences between the treatments after a Student's t-test (α=0.05). Error bars represent SE.

6.4.2.2 Blumeria graminis

Leaves of seedlings (n=12) which had been primed with Z-3-HAC showed a higher number of colonies, compared to the leaves of control seedlings (n=12) (P<0.001) (Figure 6-4A). Additionally, we observed in the control seedlings, that the sites at which the fungal colonies were forming, remained green, whereas the other areas became chlorotic. To investigate whether this effect was an artefact inherent to the bio-assay or if this could be attributed to an effect of the infection of *B. graminis*, we repeated this experiment on intact seedlings. Results from the detached leaf assay were confirmed in the new experiment: priming by Z-3-HAC resulted in a higher susceptibility to *B. graminis* (Figure 6-4B). No chlorotic regions were observed on the intact seedlings, indicating that the chlorotic areas were an artifact from the detached leaf bio-assay.

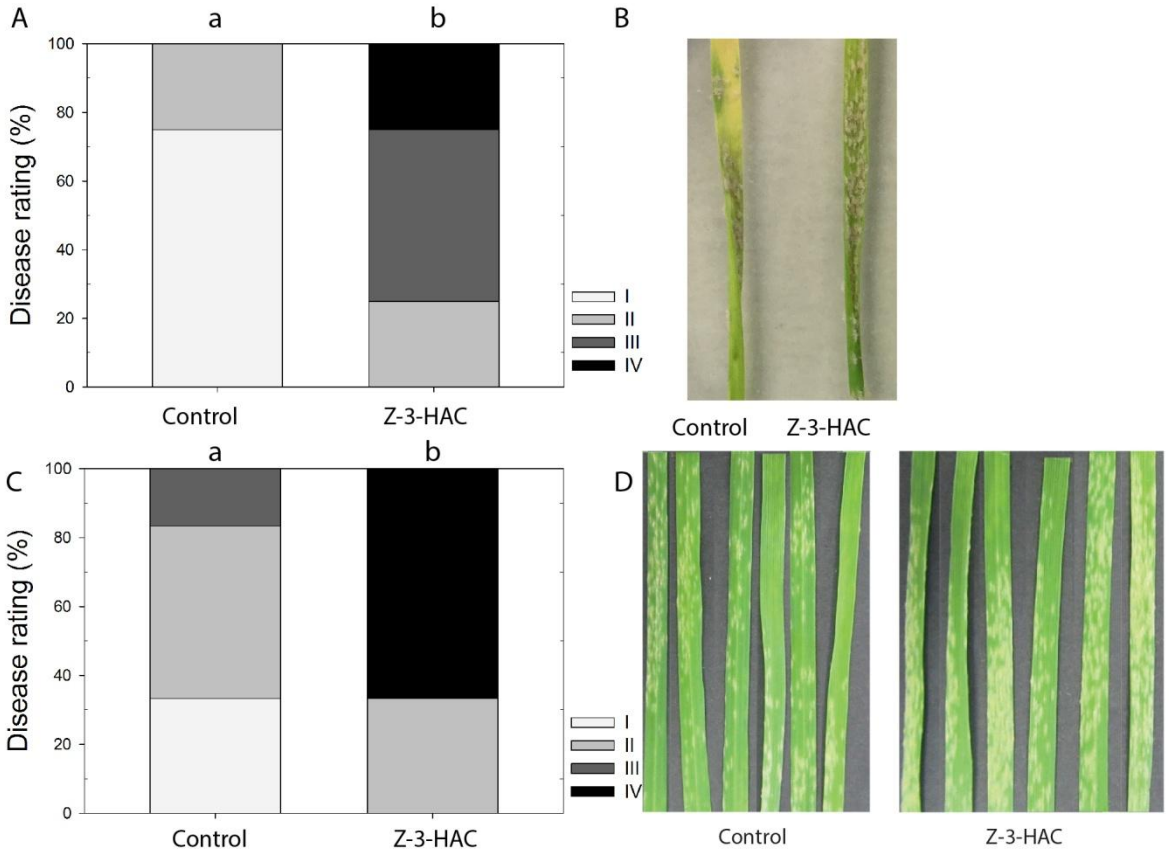


Figure 6-4: Pre-exposure of wheat to Z-3-HAC leads to a higher susceptibility to *Blumeria graminis.* A: leaves from control and Z-3-HAC-treated seedlings were cut from the plant and placed on 1.5% agar, amended with benzimidazole. Using an inoculation tower, *B. graminis* spores were dispersed uniformly over the leaves. According to the amount of infection, scores (with an ordinal scale of 1 to 4) were assigned to the different leaves (Troch *et al.*, 2014). Statistical differences were calculated using the Kruskal-Wallis test (α=0.05, n=12). B: Photographs of detached leaf bio-assay.Z-3-HAC-treated leaves remained green (right), whereas control leaves (left) became chlorotic, with the exception of sites of colonization, which remained green. C: intact seedlings from control and Z-3-HAC-treated seedlings were inoculated, using an inoculation tower. *B. graminis* spores were dispersed uniformly over the leaves. According to the amount of infection, scores (with an ordinal scale of 1 to 4) were assigned to the different leaves (Troch *et al.*, 2014). Statistical differences were calculated using the Kruskal-Wallis test (α=0.05, n=6). D: photographs of the bio-assay using intact seedlings. The experiment was done twice independently in time. Data represent the results of one experiment.

6.4.2.3 Cochliobolus sativus

Leaves of wheat seedlings which were pre-treated with Z-3-HAC (n=18) showed a lower percentage of infected area at 72 hai compared to leaves of control seedlings (n=18) (36% vs. 53%, P<0.05) after inoculation with *C. sativus* (Figure 6-5).

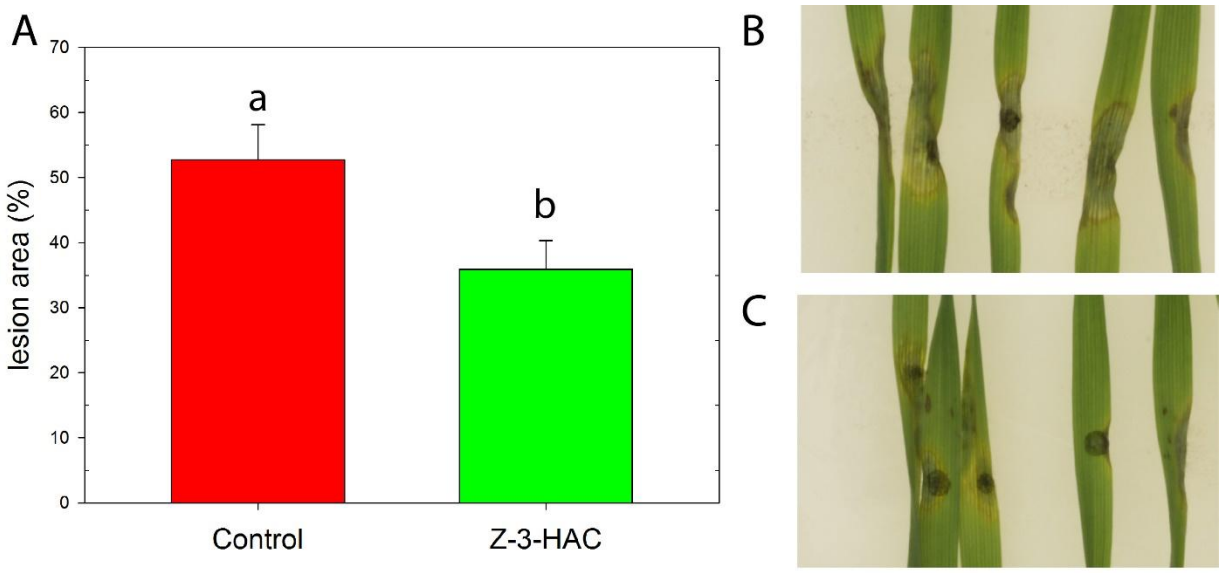
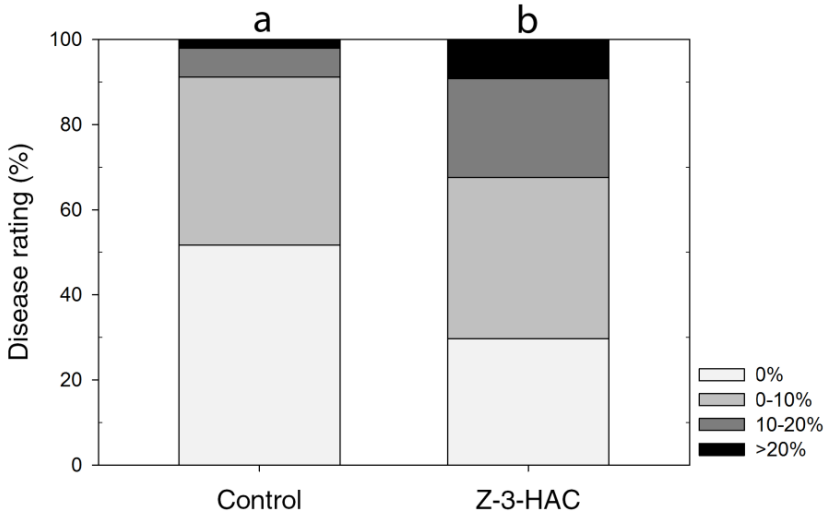


Figure 6-5: Leaves of seedlings pre-exposed to Z-3-HAC hai show smaller lesions compared after infection with *C. sativus*. Leaves were cut from the seedlings and inoculated with a conidia suspension of *C. sativus* (5 x 10^4 conidia mL^{-1}). Lesion area was monitored at 72 hai. Bars represent means of 18 biological replicates. The experiment was done twice independently in time. Data represent the results of one experiment. Bars depicted with different letters indicate significant differences between the treatments (α=0.05). Error bars represent SE. Significant differences were calculated using Student's t-test. B and C: Photographs of control-, and Z-3-HAC-treated leaves, respectively 72 hours after inoculation with *C. sativus*.

6.4.2.4 Zymoseptoria tritici

To test the effect of Z-3-HAC on the resistance of wheat against the hemibiotrophic fungus *Z. tritici*, we pre-treated wheat seedlings with Z-3-HAC and inoculated both the treated (n=90) and control group (n=90) with *Z. tritici*. After 28 days we observed in the Z-3-HAC treatment that a significant number of plants exhibited a higher susceptibility (P<0.05) compared to control plants (Figure 6-6).

Figure 6-6: Pre-exposure of wheat to Z-3-HAC leads to higher susceptibility to *Zymoseptoria tritici*. Wheat seedlings were treated with Z-3-HAC at the three leaf stadium and subsequently inoculated with a conidia suspension of *Z. tritici* (10^6 conidia mL^{-1}). After 28 days, the percentage of diseased area was determined. Data represents pooled data of two separate experiments (n=90). Significance of differences between the distribution of the classes were calculated using the Kruskal-Wallis test (α=0.05). The experiment was done twice independently in time. Data represent the results of one experiment.



6.4.2.5 Spodoptera exigua

To investigate whether Z-3-HAC-treated wheat seedlings delay the growth of *S. exigua* caterpillars, we placed freshly treated leaves on a daily basis in Petri dishes containing caterpillars. Leaves which were not eaten from the day before were removed. We performed this experiment twice, once with three Petri dishes, containing five caterpillars each (n=15), and a second time with 10 caterpillars for each Petri dish (n=30). Each experiment yielded the same results. Namely, caterpillars which were fed leaves which were treated with Z-3-HAC did not show a significant effect on growth (data not shown) or on development, compared to caterpillars which were fed non-treated leaves (Figure 6-7). However, survival was higher in caterpillars fed with Z-3-HAC treated leaves (P<0.001).

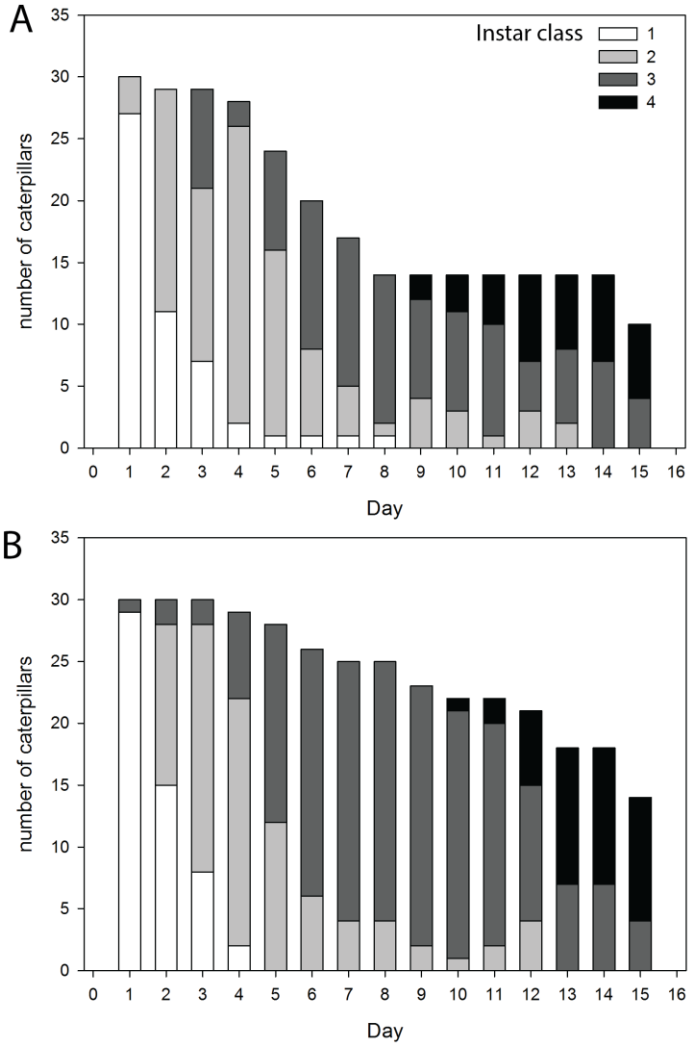


Figure 6-7: Pre-treatment of wheat seedlings with Z-3-HAC does not influence the development of *S. exigua* caterpillars (n=30). Leaves which were exposed overnight to Z-3-HAC were fed to *S. exigua* caterpillars on a daily basis. Each day, the mean weight and developmental stage of the caterpillars were recorded. The distribution of the developmental stage *of S. exigua* was not significantly different between caterpillars fed with control leaves (A) and leaves pre-treated with Z-3-HAC (B). Significance of differences between the distribution of the classes at different time points was calculated using a Kruskall-Wallis-test (α=0.05). The different colors represent the different instar classes. Data are representative for two separate experiments in time with similar results.. Significance of differences of survival between the control and Z-3-HAC treated leaves caterpillars was calculated using the Kaplan-Meier test (α=0.05). The experiment was done twice independently in time. Data represent the results of one experiment.

6.4.3 Rice

6.4.3.1 Cochliobolus miyabeanus

To investigate whether priming by Z-3-HAC resulted in increased resistance against *C. miyabeanus* infection, we analyzed the lesion area (n=11) and the number of lesions (n=6) at 72 hai in two separate experiments. Both experiments revealed that Z-3-HAC did not have an influence on both parameters (Figure 6-8 A and B).

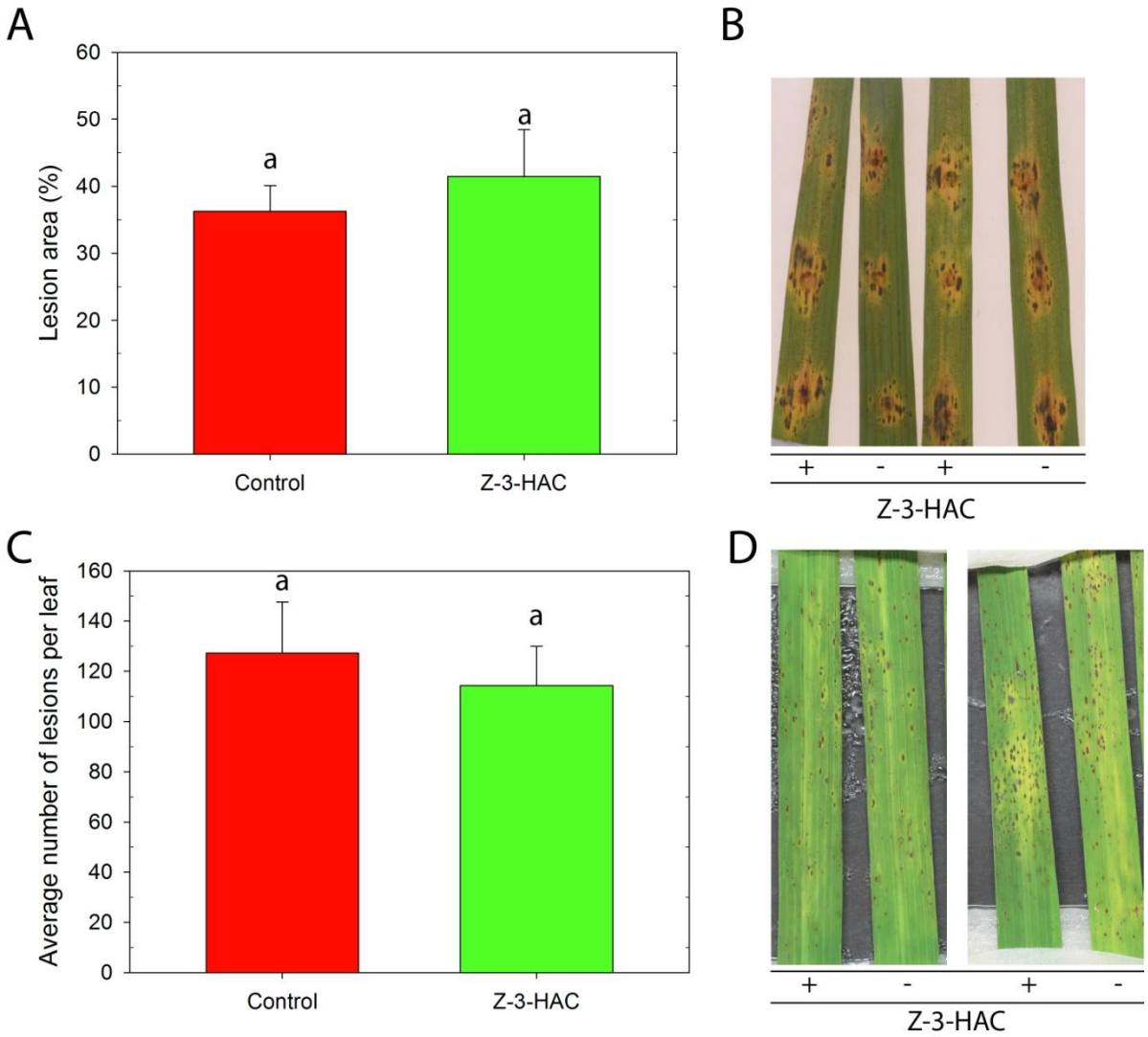


Figure 6-8: Pre-treatment with Z-3-HAC does not impact the lesion area (A) or number of lesions (C) in rice after inoculation with *Cochliobolus miyabeanus*. Rice plants were pre-treated with Z-3-HAC overnight. The following day, leaves were cut off and subsequently inoculated with three droplets of a conidia suspension (1 x 10^4 ml^{-1}) per leaf (A) or with a spray inoculation (C). Bars represent means of 11 biological replicates (A) and 6 biological replicates (C). Bars depicted with different letters indicate significant differences between the treatments. The experiment was done twice independently in time. Data represent the results of one experiment. Error bars represent SE. Significance of differences was calculated using Student's t-test (α=0.05). B and D: Photographs of the droplet, and spray inoculation, respectively.

6.4.3.2 Magnaporthe oryzae

To evaluate the efficacy of Z-3-HAC in enhancing resistance against *M. oryzae*, we pre-treated rice plants and inoculated them with a conidia suspension. After 6 days, we counted

the number and type of lesions (resistant vs. susceptible). Rice seedlings which had been primed with Z-3-HAC (n=9) showed a 41% reduction (P<0.01) in the ratio of susceptible to resistant lesions, compared to the control (n=9).

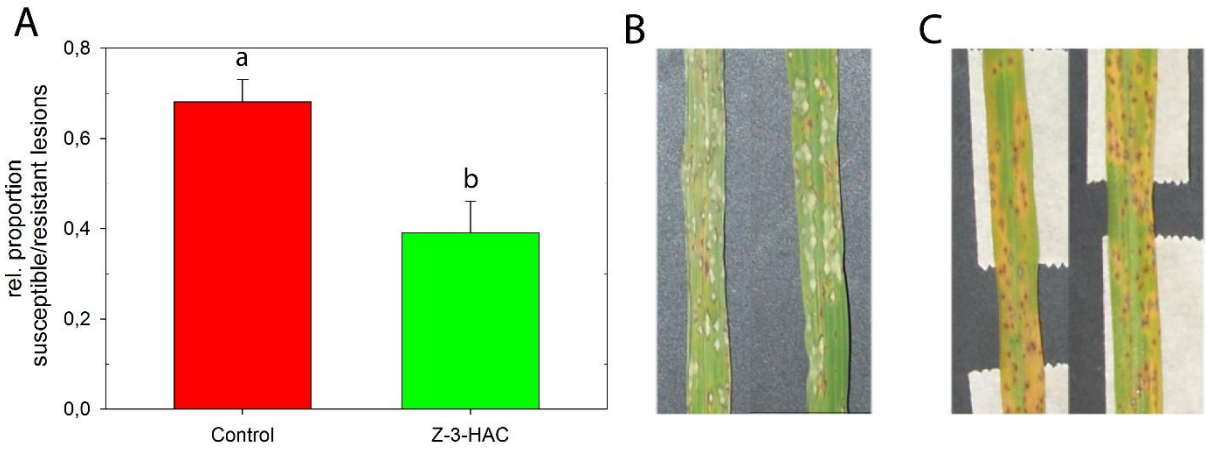


Figure 6-9: Pre-treatment with Z-3-HAC results in relative more susceptible lesions in rice after inoculation with *Magnaporthe oryzae*. A: Five-week old rice plants were treated overnight with Z-3-HAC and the following day inoculation with a spore suspension of *M. oryzae*, using an airbrush connected to an air compressor. After 6 days, the lesions and type of lesions were recorded. Bars represent means of 9 biological replicates. Error bars represent SE. Different letters indicate significant differences between the treatments after a Student's t-test (α=0.05). B: leaves of the control treatment show more lesions with a gray centre, indicative of sporulation. C: leaves of the Z-3-HAC treated leaves show more lesions typical of a resistant response. The experiment was done twice independently in time. Data represent the results of one experiment.

6.4.3.3 Xanthomonas oryzae

Priming by Z-3-HAC did also result in enhanced resistance in rice seedlings against *Xoo*. Lesion length after 14 days was 35% shorter in primed seedlings (n=10) compared to control seedlings (n=10) (P<0.05) (Figure 6-10A). CFU's were lower in the Z-3-HAC treated plants, but differences were not significant (Figure 6-10B).

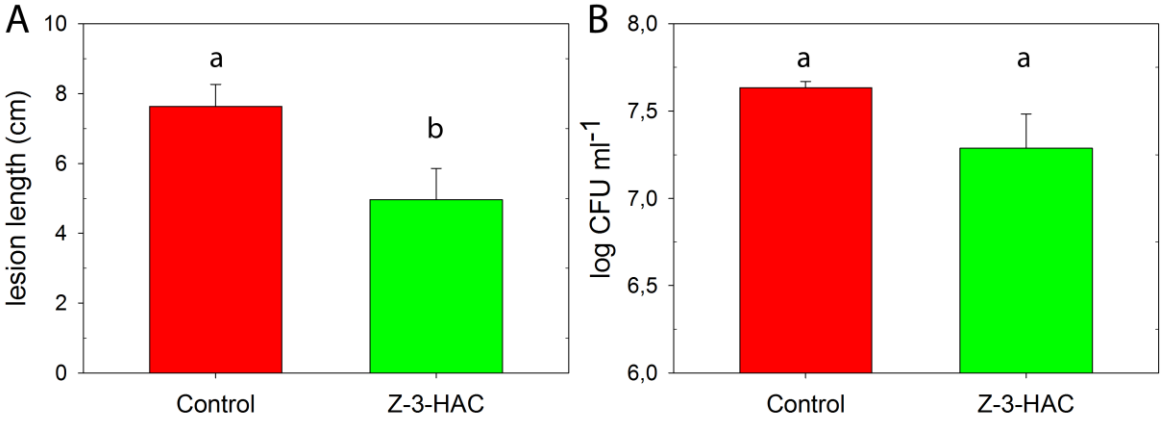


Figure 6-10: Pre-exposure to Z-3-HAC results in smaller lesions of *X. oryzae pv. oryzae* (Xoo). Rice seedlings (n=10) were pre-exposed overnight to Z-3-HAC and inoculated with a bacterial suspension (OD_{620}=0.5) of *Xoo*. Two weeks after inoculation, lesions and CFU's were determined. The experiment was done twice independently in time. Data represent the results of one experiment. Error bars represent SE. Different letters indicate significant differences between the treatments. Significant differences were calculated using Student's t-test (α=0.05).

6.4.3.4 Rhizoctonia solani

Lesions were 38% smaller (P<0.05) in rice plants which were treated with Z-3-HAC (n=7) compared to control plants (n=8) after inoculation with *R. solani* (Figure 6-11).

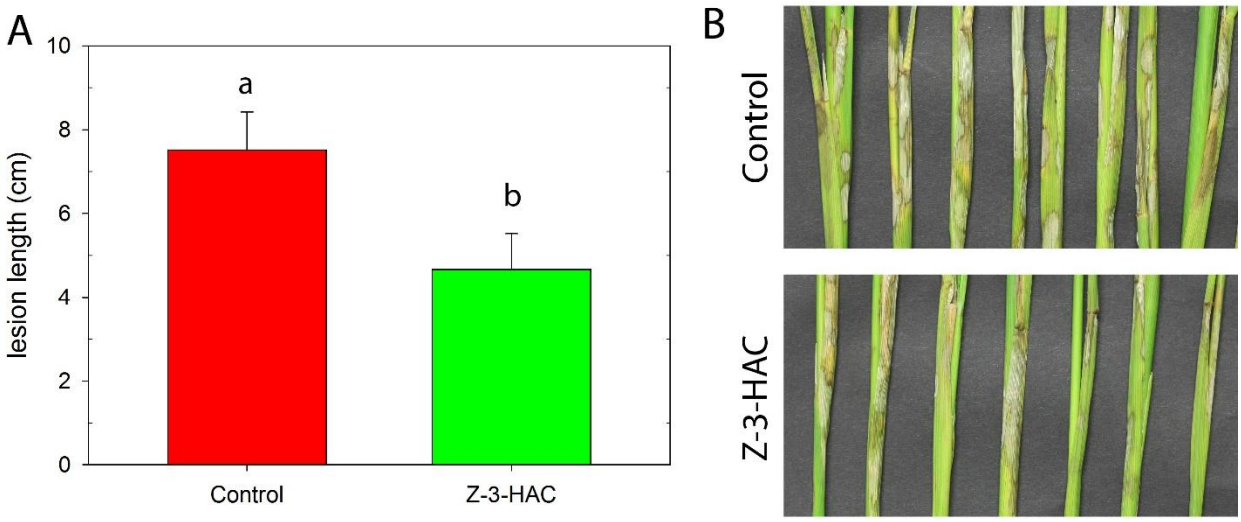


Figure 6-11: Treatment with Z-3-HAC results in a lower lesion length in rice after inoculation with *Rhizoctonia solani*. Rice plants were treated overnight with Z-3-HAC and the following day, a tooth pick colonized with *R. solani* was inserted in the leaf sheath of the second youngest leaf. After three days, the lesions were measured. A: Bars represent means of seven biological replicates. The experiment was done twice independently in time. Data represent the results of one experiment. Error bars represent SE. Different letters indicate significant differences between the treatments after a Student's t-test (α=0.05). B: Photographs of the control (top) and Z-3-HAC-treated (bottom) leaf sheaths three days after inoculation.

6.4.3.5 Meloidogyne graminicola

To examine whether priming by Z-3-HAC also influenced resistance of rice roots against infestation by the root knot nematode *M. graminicola*, we inoculation roots with a nematode suspension and evaluated the number of galls and development of the nematodes after 14 days. Leaf and root length was not significantly different between control and Z-3-HAC treated plants (data not shown); demonstrating that Z-3-HAC did not influence leaf, or root growth. However, the number of galls was significantly lower in Z-3-HAC treated plants (n=10) compared to the control plants (n=10) (-27.6%, P < 0.05). However, the development of *M. graminicola* did not show a difference between the control and Z-3-HAC treatment (P=0.18).

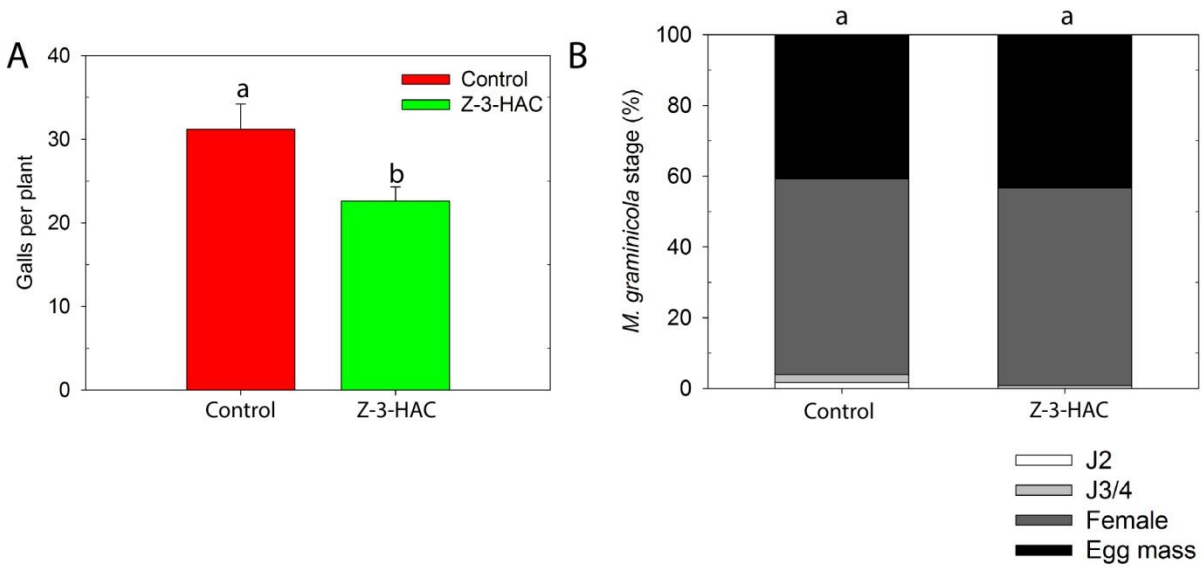


Figure 6-12: Pre-exposure to Z-3-HAC reduces the number of galls (A) in rice but has no influence on the nematode development of *M. graminicola* (B). Two-week-old rice plants (n=10) were pre-treated with Z-3-HAC and the following day inoculated with 200 nematodes per plant. Two weeks after inoculation, the number of galls and the developmental stage of the nematodes were recorded. Different letters above the bars indicate significant differences between the treatments. The experiment was done twice independently in time. Data represent the results of one experiment. Significance of differences was calculated using Student's t-test for A and the Kruskall-Wallis test for B (α=0.05)**.**

6.5 Discussion

6.5.1 Z-3-HAC does not prime for enhanced resistance in each pathogen-plant interaction

The results from Chapter 4 revealed that Z-3-HAC primed wheat seedlings for enhanced resistance against *F. graminearum*. Results from follow-up experiments pointed to a model in which Z-3-HAC primes wheat plant for enhanced JA dependent defense. In addition, in Chapter 5, we found evidence for increased SA biosynthesis upon Z-3-HAC exposure. However, in this chapter we performed measurements of SA and JA of rice seedlings and found contrasting results. Namely, in rice SA levels were not influenced upon Z-3-HAC exposure, whereas JA levels were higher in Z-3-HAC treated plants (Figure 6-2).

The role of SA in rice has remained enigmatic in the past as no effects on SA levels were found upon infection with pathogens (Silverman *et al.*, 1995; Iwai *et al.*, 2007). However, as BTH acts downstream of SA biosynthesis and confers resistance, it has been suggested by De Vleesschauwer *et al.* (2014), that it is rather the signaling action of SA that mediates defense during plant-pathogen interaction, than SA biosynthesis itself.

To investigate whether Z-3-HAC primes defense against pathogens against which defense is known to be governed by SA and/or JA dependent defense, we performed several inoculation experiments. In short, we found that Z-3-HAC enhanced wheat resistance against

Fusarium spp. (Figure 6-3) and *C. sativus* (Figure 6-5), had no effect on the growth and development of *S. exigua*, but decreased mortality (Figure 6-7), and induced susceptibility against *B. graminis* (Figure 6-4) and *Z. tritici* (Figure 6-6). For rice, we also found different outcomes for the different pathosystems: Z-3-HAC enhanced resistance against *M. oryzae* (Figure 6-9), *X. oryzae, pv. oryzae* (Figure 6-10), *R. solani* (Figure 6-11) and the root knot nematode *M. graminicola* (Figure 6-12), but had no effect on resistance against *C. miyabeanus* (Figure 6-8).

While a detailed investigation of involved defense pathways is outside the scope of this chapter, we can survey studies investigating defense pathways for the different pathosystems (Figure 6-13).

6.5.2 Z-3-HAC influences wheat resistance to fungal pathogens in a pathogen-specific manner

The ***F. graminearum***-wheat experiment confirmed our results previously reported in this doctoral thesis (section 4.4.1). Namely, pre-treating plants with Z-3-HAC resulted in smaller lesions compared to control plants. This reduction in lesion length was also apparent for *F. poae*, another member of the FHB disease complex. Additionally, inoculation with ***F. poae***, did not result in necrotic colonization of the leaf and spread from the initial inoculation site was limited. However, a reduction in lesion size was observed for Z-3-HAC treated plants compared to the control leaves (Figure 6-3). The lack of aggressiveness for *F. poae* has already been reported in other studies (Vogelgsang *et al.*, 2008; Xu *et al.*, 2008) and it has been suggested that due to this response, *F. poae* more likely acts as a secondary invader of wheat (Vanheule, 2016). The role of JA and SA has already been discussed in section 4.5.2, in which we observed a clear biphasic response in which SA confers resistance in the biotrophic phase of the infection by *F. graminearum* and JA confers resistance in the necrotrophic phase.

We also tested resistance against ***B. graminis*** in wheat, which has an obligate biotrophic lifestyle. Even though JA and SA have been reported to be positive inducers of defense against *Blumeria* in wheat (Beßer *et al.*, 2000; Walters *et al.*, 2002; Duan *et al.*, 2014), more colonies were formed on Z-3-HAC treated seedlings, compared to control seedlings (Figure 6-4). It has recently been shown in Desurmont *et al.* (2016) that GLV production of caterpillar infested plants was less in *B. rapa* plants after *B. graminis* infection compared to non-infected plants. The lower GLV production in these plants indirectly suggests that plants lower GLV production in the defense against *B. graminis* and consequentially that exogenously applied Z-3-HAC may promote infection. Furthermore, in our detached-leaf experiment we found green patches surrounding the places where colonies were forming,

whereas the other areas became chlorotic (Figure 6-4B). It has already been described that *B. graminis* produces cytokinins which delay senescence in leaves and rewire plant resources to keep plant cells alive, resulting in so called “green islands” (Walters *et al.*, 2008).

A third pathogen we tested was ***C. sativus*****,** which is considered a hemi-biotrophic pathogen, entailing that defense against *C. sativus* also occurs in a biphasic manner. To our knowledge, there are only limited studies available that indirectly implicate JA in the defense against *C. sativus*, and SA to a lesser extent (Dong *et al.*, 2010; Zhang *et al.*, 2012). The involvement of JA in the defense against *C. sativus* is in accordance with our model from Chapter 4, that Z-3-HAC increased JA dependent defense, resulting in smaller lesions in our infection assay (Figure 6-5).

Our infection assay with *Z. tritici* showed a higher susceptibility after treatment with Z-3-HAC (Figure 6-6). Infection of wheat leaves by ***Z. tritici*** is characterized by a long biotrophic phase, in which the fungus grows intercellularly, which can last up to 10 days. This phase is followed by a rapid switch to the necrotrophic phase of which the exact mechanism remains unknown (Gohari *et al.*, 2015; Rudd *et al.*, 2015). Early stages of the infection coincide with a suppression of PR proteins and JA biosynthesis genes while at the onset of the necrotrophic/symptomatic stage an upregulation of JA biosynthesis genes and a strong increase in SA levels was reported (Yang *et al.*, 2013; Rudd *et al.*, 2015). This suggests that Z-3-HAC activated signaling pathways which in turn suppresses defense. Namely, Z-3-HAC may have counteracted the early JA biosynthesis gene suppression or the later SA accumulation preluding the necrotrophic stage (Rudd *et al.*, 2015). However, our phytohormone analysis revealed a strong increase in SA levels in primed plants at early time points, which contradicts this hypothesis (Figure 5-8). As pathogenesis is the result of the interplay between plant defense and pathogen infection strategies, the tampering of the pathogen with the host’s defense might provide some answers to the increased susceptibility.

6.5.3 Insects

While fungal pathogens are more restricted within the plant tissue and thus need a more sophisticated infection mechanism, chewing insect herbivores on the other hand employ a cruder method of feeding. To investigate whether Z-3-HAC exposed leaves are more detrimental to ***S. exigua*** growth and development, we fed freshly treated leaves to *S. exigua* caterpillars. After monitoring for two weeks, no influence of Z-3-HAC on the growth or development of caterpillars between the two treatments was found. On the other hand, a higher mortality was observed in the control leaves. This was unexpected as an activation of JA and ET dependent defenses have numerously been reported to exert negative effects on

Spodoptera spp. in dicots and monocots (Thaler *et al.*, 2002a; Xu *et al.*, 2003; Kessler *et al.*, 2004; Shivaji *et al.*, 2010; Christensen *et al.*, 2013a). Besides JA, the defense hormone SA has only indirectly been shown to negatively influence maize defense by inhibiting JA signaling (Shivaji *et al.*, 2010). One of the defense mechanisms of plants against insects is the production of proteinase inhibitors (**PIs**) (War *et al.*, 2012). These play an important role in the defense of plants against insects by inhibiting proteases of insects and thus providing antinutrional effects (Zhu-Salzman & Zeng, 2015). The link between JA and PI's has already long been known (Farmer *et al.*, 1992; Farmer & Ryan, 1992) whereas a link between PI and Z-3-HAC is undisclosed, Farag *et al.* (2005) found contrasting results between the GLV Z-3-HOL and PI production in maize. Even though they found an increase of transcripts of maize proteinase inhibitor, this did not result in increased PI products. The latter study and our results do not provide evidence that GLVs increased plant resistance by impeding *Spodoptera* growth or development. On the contrary, it seems that Z-3-HAC treated leaves benefited the caterpillars. In parallel to the *B. graminis* pathosystem, Z-3-HAC may have increased endurance strategies, resulting in a rewiring of plant resources. Additionally, the strong increase in SA levels, may have inhibited JA dependent defense responses, leading to decreased production of PIs. We believe more research on the nutritional values of GLV treated leaves is necessary to solve this question. It has been shown by Halitschke *et al.* (2004a) and Meldau *et al.* (2009) that GLVs may serve as feeding stimulants. It remains possible that our Z-3-HAC treatment induced GLV production in wheat leaves as was seen in maize (Engelberth *et al.*, 2004), which led to increased consumption by the caterpillars. It does however remain enigmatic how this led to an increase in survival but not in caterpillar biomass or development. However, it has been raised by Mewis *et al.* (2005) that different means of assessing plant resistance against insects can lead to very different results. Namely, studies which gave the choice between a control and treated plant material cannot be compared to studies which only presented caterpillars with one type of food as compensatory feeding can occur. This entails that insects which were fed with low-quality plants might consume more of the plant to acquire the same level of nutrients. Thus, higher weights gains, do not necessarily indicate healthier caterpillars. Our experimental design did not allow the caterpillars a choice between control and Z-3-HAC treated leaves, thus possibly confounding conclusions from our experiment. Additionally, as freshly primed leaves were supplied to the caterpillars, we were not able to observe whether late Z-3-HAC induced responses (e.g. 2 days after exposure) might have affected caterpillar growth and survival differently. In the future, the use of bigger plants may allow us to monitor the amount of leaf area that is consumed each day. The optimization of our feeding assay is necessary to better understand the interaction between Z-3-HAC-primed wheat and caterpillar performance.

6.5.4 Z-3-HAC influences rice resistance in a similar fashion to JA

C. miyabeanus is the causal agent of brown spot disease in rice. While for *C. sativus* studies only indirectly demonstrate the involvement of defense hormones, for *C. miyabeanus* more information is available. In the study of De Vleesschauwer *et al.* (2010) no effect of JA, BTH or BABA was found on the resistance against *C. miyabeanus*. These results corroborate the findings of Ahn *et al.* (2005) which also did not find increased resistance after treatment with BTH or MeJA. Results from our experiments are in line with these findings, as we also found no effect of pre-treatment of Z-3-HAC on the disease severity (Figure 6-8), whereas we increased JA production after Z-3-HAC treatment (Figure 6-2).

M. oryzae is a hemi-biotrophic fungus causing blast in rice. We found a significant lower proportion of susceptible lesions in Z-3-HAC treated plants (Figure 6-9). While SA has been reported to contribute to resistance against *M. oryzae* (Ahn *et al.*, 2005; Iwai *et al.*, 2007; Shimono *et al.*, 2007), a role for JA in the resistance against blast is more ambiguous. Ahn *et al.* (2005) found that treatment with MeJA was ineffective. However the study from Riemann *et al.* (2013) found that knockout mutants of *OsAOC*, a gene involved in the biosynthesis of JA, were more susceptible to *M. oryzae* infection, demonstrating that JA is involved in the infection process *M. oryzae*. This subscribes an earlier study of Mei *et al.* (2006), in which they overexpressed *OsAOC2* genes in rice, under the control of a strong pathogen-inducible promotor. These mutants accumulated higher levels of JA and enhanced activation of PR genes (*PR1*, *PR3* and *PR5*) after infection with *M. oryzae*. On the other hand, using RNAi, expression of *AOC* and *OPR* was suppressed in rice, which did not lead to differences in resistance to *M. oryzae*, compared to WT plants (Yara *et al.*, 2008). Because of the induction of higher JA levels, and increased resistance in Z-3-HAC treated leaves, this advocates in favor of a protective function of JA in the rice-*M. oryzae* interaction.

In our study, we also included the hemibiotrophic bacterial pathogen ***Xoo,*** causing bacterial leaf streak. The JA signaling pathway is involved in *Xoo* resistance, as exogenously administered JA leads to smaller lesions (Yamada *et al.*, 2012). Furthermore, the JA inducible production of the monoterpene alcohol linalool is known to induce resistance against *Xoo*. The same study found that overexpression of linalool synthase coincided with increased PR gene expression (Taniguchi *et al.*, 2014). Our infection assay revealed a protective role for Z-3-HAC against *Xoo* (Figure 6-10), which is in agreement with the increased JA production, which has been reported to confer resistance to *Xoo*. On the other hand, BTH, OsNPR1 and OsWRKY45 overexpression have been implicated in resistance against *Xoo* as well, demonstrating that the SA signaling pathway is critical for resistance against *Xoo* (Nakashita *et al.*, 2003; Yuan *et al.*, 2007; Shimono *et al.*, 2012). Two pairs of allelic WRKY45 genes have opposite roles in resistance against *Xoo* in rice. OsWRKY45-2

acts as a positive regulator of resistance against *Xoo* and *M. oryzae* and is found in the *indica* rice, while OsWRKY45-1 confers susceptibility towards *Xoo*, and is found in *japonica* rice (Tao *et al.*, 2009). They also observed that OsWRKY45-2-mediated resistance was accompanied by increased expression of *OsWRKY13* and concomitant expression of *PR1a* and *PR1b*. OsWRKY13 has been implicated in the resistance against *Xoo* and *M. griseae*. OsWRKY13 functions as gateway between JA-SA signaling: it activates SA-dependent defenses, while at the same time repressing expression of JA biosynthesis genes (Qiu *et al.*, 2007; Qiu *et al.*, 2008). OsWRKY13 has been shown to be activated by both SA and JA and to bind to the two alleles of OsWRKY45 and thus might function as a transcriptional repressor of OsWRKY45 (Qiu *et al.*, 2009). The increase in JA upon Z-3-HAC exposure may have upregulated OsWRKY13 expression, which in turn downregulates OsWRKY45-1, resulting in increased resistance.

Whereas aforementioned fungi are considered (hemi-)biotrophs, ***R. solani***, causing sheath blight disease in rice, has generally been accepted to be a necrotrophic pathogen. Taheri & Tarighi (2010) showed that riboflavin acted as a defense activator in rice against *R. solani* by activating the expression of LOX and priming for increased lignification. The role of JA in the resistance was confirmed by exogenous JA application and use of JA deficient mutants and a LOX inhibitor. Much less is known about the role of SA signaling in *R. solani* resistance. While BTH has been reported to induce resistance against *R. solani* (Rohilla *et al.*, 2002), overexpression of WRKY45 did not lead to increased resistance (Shimono *et al.*, 2012). In our experiments, we found increased resistance against *R. solani* after treatment with Z-3-HAC.

We were interested whether treatment with Z-3-HAC would also result in enhanced resistance in the roots of rice against the obligate biotrophic root knot nematode ***M. graminicola***. Surprisingly, we found that aerial treatment of the leaves also conferred enhanced resistance in the roots. We observed a lower number of galls in Z-3-HAC treated plants, and no effect on the development (Figure 6-12). These results suggest that the initial colonization by the nematodes in the roots is impeded in Z-3-HAC treated plants, but that after colonization, there is no effect on the development of nematodes. In rice roots, defense is predominantly driven by SA and JA dependent defenses, whereas the other hormones (ABA, BRs, auxins, CKs and GAs) do not contribute much to immunity (Kyndt *et al.*, 2014).

JA is known to play a pivotal role in the defense of rice against *M. graminicola*. During its early infection process, the nematodes will suppress SA and JA defense to facilitate infection (Kyndt *et al.*, 2014; Kumari *et al.*, 2016; Mantelin *et al.*, 2017). This is subscribed by an earlier study in which JA was exogenously applied and resulted in enhanced resistance against *M. graminicola*, by counteracting the defense suppression of the nematode (Nahar *et al.*, 2011).

6.6 Concluding remarks

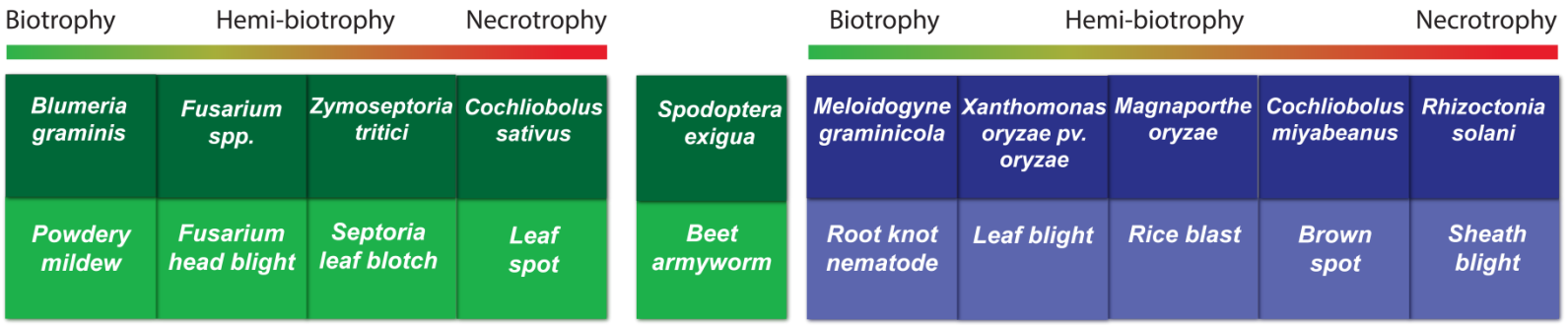
In summary, we have found that treating wheat and rice with the GLV Z-3-HAC does not confer global defense enhancement. While wheat became more resistant against *F. graminearum* and *F. poae*, an adverse effect was observed for *B. graminis* and *Z. tritici*. Rice became more resistant against *M. oryzae, X. oryzae pv. oryzae, R. solani* and *M. graminicola*, but no effect was found against *C. miyabeanus*. Whereas GLV treatment has generally been associated with activation of JA dependent defense, we were not able to discern a clear link between Z-3-HAC and plant resistance against pathogens which are known to be mediated by JA signaling in wheat. While we observed in Chapter 5 that Z-3-HAC boosts SA production in wheat, this did not always result in enhanced defense in pathosystems against which defense is mediated by SA. This may suggest that Z-3-HAC-mediated signaling occurs in a JA- and SA independent manner or that the timing of the Z-3-HAC treatment plays a critical role (section 4.4.3).

On the other hand, for rice our phytohormone analysis revealed an increase of JA after Z-3-HAC exposure. In our infection assays in which resistance against the pathogen is known to be mediated by JA, we found also for Z-3-HAC treated seedlings increased resistance (with the exception of *Xoo*). These results suggest that for rice, increased resistance after Z-3-HAC exposure is governed by enhanced JA dependent defense.

Another hypothesis is that Z-3-HAC activates processes which aim at keeping cells alive and evade PCD and senescence (cfr. endurance strategy, section 3.6). Indeed, fungi with an obligate biotrophic lifestyle (*B. graminis*) or an outspoken long biotrophic phase (*Z. tritici*) were more virulent on Z-3-HAC treated plants.

In order to get a better grasp on the involved signaling pathways in the different pathways, use of knockout and overexpressing mutants is indispensable and a more holistic approach is necessary. For example, a transcriptomics study of the different pathosystems may reveal nodes of convergence in defense signaling pathways which might integrate the results of our different infection assays.

Overall, these results indicate that Z-3-HAC may constitute a promising tool in the biocontrol against a broad range of pathogens. On the other hand, besides the risk of increased DON contamination (Chapter 4), we have shown that Z-3-HAC induced susceptibility in wheat against *B. graminis* and *Z. tritici*. Thus, application of Z-3-HAC entails the risk of making wheat more susceptible to those pathogens.



Defense Hormone										
SA	+	+	+	+	-	(-)	+	+	(-)	(-)
JA	+	+	+	+	+	+	+	+	(-)	+
Z-3-HAC	-	+	-	+	(-)	+	+	+	(-)	+

Figure 6-13: Overview of the effect of Z-3-HAC on the different tested pathosystems amended with known interactions of phytohormones with those pathosystems. For *S. exigua*, we do not have data on plant damage. Hence, the results of Z-3-HAC exposure against *S. exigua* have been shown as (-). Pathogens of wheat are shown in green boxes, whereas pathogens of rice are shown in blue boxes. Defense hormones which increase resistance against a specific pathogen or insect are depicted with '+', increased susceptibility: '-', no effect: '(-)'. Abbreviations: SA, salicylate, JA, jasmonate; Z-3-HAC, Z-3-hexenyl acetate. See the text for references used to construct the table. Adapted from De Vleesschauwer *et al.* (2013).

Chapter 7 General Discussion - Future Perspectives

7.1 General discussion

7.1.1 Introduction

The main hypothesis of this doctoral thesis was that **exposure to GLVs primes defense responses in wheat in the defense against fungal pathogens**. Sub-hypotheses were that **priming by GLVs is mediated by JA dependent signaling** and that **priming may confer increased susceptibility in other pathosystems**. To test these hypotheses, we addressed different research questions. In the introductory chapters, we provided a state of the art of plant defense and priming (**Chapter 2**), with a focused view on GLVs (**Chapter 3**). In **Chapter 4**, we used the wheat - *F. graminearum* model system to investigate the priming potential of the green leaf volatile Z-3-HAC and get a first glimpse at the underlying priming mechanisms. To obtain a better and more holistic understanding of the wheat response upon Z-3-HAC exposure, we performed an untargeted metabolomics study of the metabolome during plant-pathogen interaction in **Chapter 5**. Lastly, we screened several pathosystems to investigate the applicability of Z-3-HAC in wheat and rice (**Chapter 6**).

7.1.2 General overview

The outcome of plant-pathogen interactions during pathogenesis is governed by an intricately timed and placed action- and reaction mechanism (Pieterse *et al.*, 2012). For plants, a timely recognition of the invading pathogen is mandatory to mount the necessary defenses in order to halt the pathogen's colonization. On the other hand, for pathogens, the avoidance of the recognition and suppression/circumvention of the plants immunity are indispensable to successfully infect the plant tissue. To achieve successful plant defense, resources will be rewired and defense genes will be upregulated. In this study, we predominantly used RT-qPCR and (un)targeted metabolomics to gain more insight in the changes in plant processes upon Z-3-HAC exposure and following *F. graminearum* infection. An overview of our findings, in the wheat-*F. graminearum* pathosystem, is visualized in Figure 7-1, and will be discussed in the different sections below.

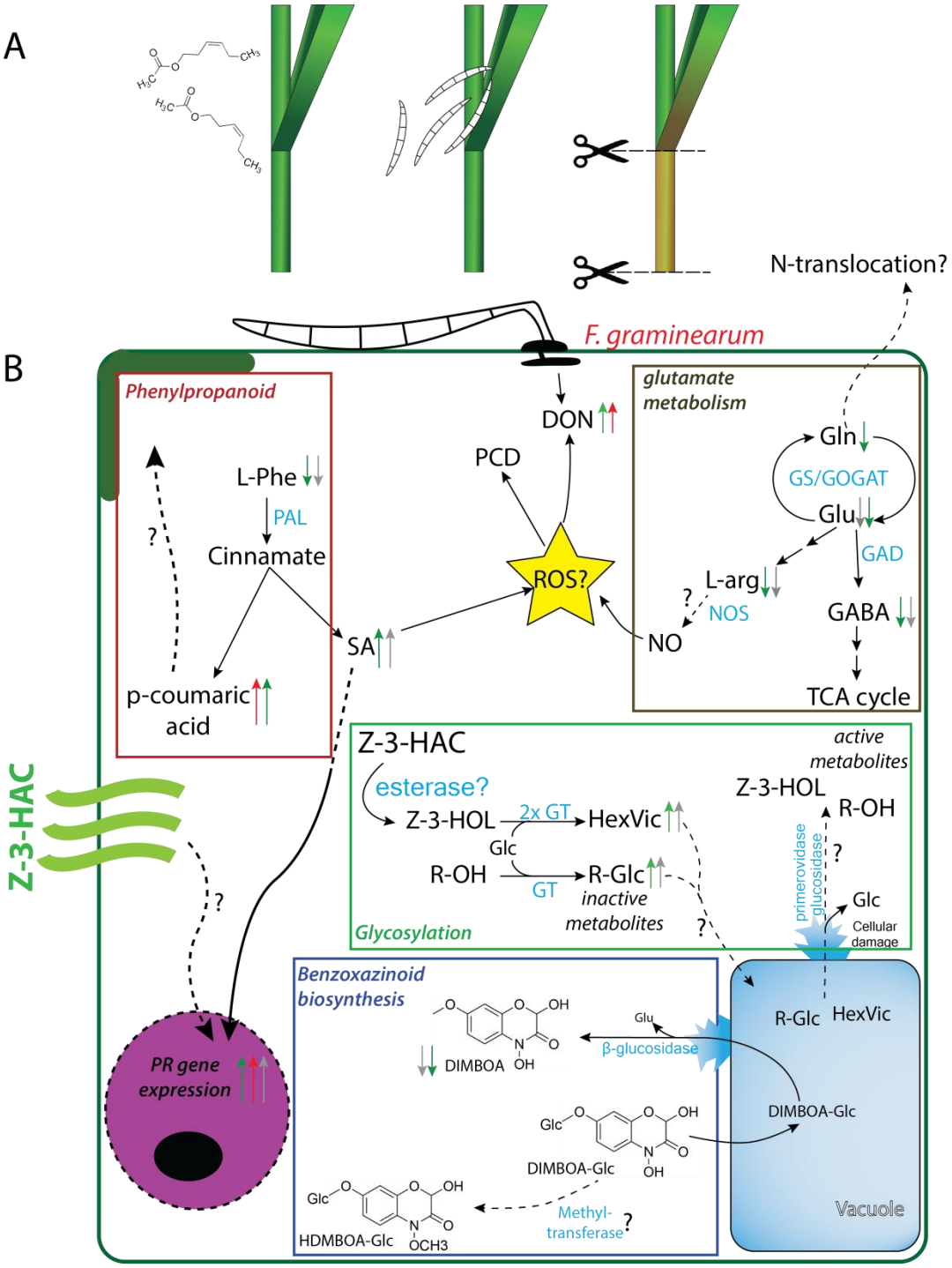


Figure 7-1: Diagram depicting the treatment and sampling method (A) and the affected pathways in plants after Z-3-HAC exposure and/or *F. graminearum* inoculation in wheat (B). Enzymes are depicted in blue, dashed arrows represent hypothetical interactions. Colored arrows besides the metabolites represent whether it was up- or downregulated in a certain treatment. The color of the arrows follows the scheme in Table 4-2; black: control, grey: Z-3-HAC; green: Z-3-HAC+*F. graminearum* inoculation; red: *F. graminearum* inoculation. L-Phe, L-phenylalanine; Gln, glutamine; Glu, glutamate; L-arg, L-arginine; GABA, γ-amino butyric acid; TCA, tricarboxylic acid; NO, nitric oxide; SA, salicylic acid; PR, pathogenesis related; Glc, glycoside; Z-3-HAC, z-3-hexenyl acetate; Z-3-HOL, z-3-hexenol; PAL, phenylalanine ammonia lyase; GS, glutamine synthetase; GOGAT, glutamine:2-oxoglutarate aminotransferase; GAD, glutamate decarboxylase; GT, glycosyltransferase; NOS, nitric oxide synthase.

7.1.2.1 Defense gene expression

Based on the RT-qPCR data from the different experiments (MeSA/MeJA/*F. graminearum* challenge), we observed that Z-3-HAC alone did not significantly affected defense gene expression. However, because of the post-hoc pairwise comparisons between the treatments, a possible effect of Z-3-HAC may have gone undetected. Indeed, after compiling data from the different RT-qPCR experiments in which we aggregated RT-qPCR data from control and Z-3-HAC treated wheat seedlings, we found significant effects on defense gene expression. Namely, Z-3-HAC significantly upregulated *PR4* (4.09-fold), *PR5* (4.77-fold) and *ICS* (4.99-fold) at 39 hours after exposure (data not shown). Pre-exposure with Z-3-HAC affected defense gene expression differently according to the following challenge. A challenge with MeSA resulted in higher expression in nonprimed leaves, whereas a challenge with MeJA resulted in a higher expression in primed leaves (Figure 4-10). The small upregulation of defense genes after Z-3-HAC exposure, followed by a stronger upregulation after MeJA treatment points to a priming response as depicted in Figure 2-4, in which Z-3-HAC enhances JA dependent responses. However, it remains unclear whether Z-3-HAC directly activates gene expression via a yet unknown signaling pathway or whether Z-3-HAC indirectly activates gene expression through interference with SA or JA dependent pathways. The role of SA and JA in the priming response is further discussed in section 7.1.3.

7.1.2.2 Phenylpropanoid pathway

The phenylpropanoid pathway is an important pathway in plants yielding metabolites involved in stress responses such as SA, flavonoids, lignin, and hydroxycinnamic acids (Dixon *et al.*, 2002). To get a first insight into the effect of Z-3-HAC on this pathway, we selected L-Phe and p-coumaric acid, which constitute two key metabolites in the phenylpropanoid pathway and the plant hormone SA. Z-3-HAC exposure clearly decreased L-Phe levels in wheat, whereas p-coumaric acid was not influenced by Z-3-HAC, indicating that L-Phe was utilized by other pathways further downstream. One possible route is the production of SA, via benzoic acid, which was up to 3.2 times higher in the Z-3-HAC treatment compared to the control. The main route for SA biosynthesis is the production via the shikimate pathway, which also produces L-Phe. Hence, the decrease in L-Phe, may also be attributed to substrate limitation of chorismate. However, this remains speculation without further investigation of the shikimate and phenylpropanoid pathway. While Z-3-HAC did not influence p-coumaric acid levels, *F. graminearum* inoculation increased p-coumaric acid levels at 24 and 48 hai. Metabolites downstream of p-coumaric acid also consist of lignins, which play important roles in cell wall fortification upon pathogen infection (Dixon *et al.*,

2002). . Histochemical staining techniques may reveal whether Z-3-HAC interferes with cell wall fortification.

7.1.2.3 Glutamate metabolism

Glutamate metabolism plays an important role in the primary C/N metabolism in plant cells. Glutamate acts a NH_4^+ acceptor and N donor for other amino acids, and through the GS/GOGAT cycle, N can be transported under the form of glutamine to distal tissue. Because of this property, glutamate metabolism has been appointed an important role in plant defense (Seifi *et al.*, 2013b).

In Chapter 5, we found decreases in glutamate and glutamine levels in the Z-3-HAC + Fg treatment, pointing to a N remobilization away from the inoculated tissue to deprive the pathogen from nutrients and induce PCD. Glutamate and glutamine levels were not significantly different in the Z-3-HAC, and *Fg* treatments, which shows that neither Z-3-HAC in itself or *F. graminearum* induced N translocation. Hence, Z-3-HAC prepares mechanisms to quickly transport N away upon recognition of a MAMP. Furthermore, the requirement of the presence of MAMPs safeguards the plant from unnecessarily inducing N translocation upon Z-3-HAC exposure.

Besides the importance of temporal aspects during plant pathogen interactions, the spatial dynamics should not be overlooked. After recognition of the pathogen, plant defenses are activated, not only locally, but also in distal tissue (see section 2.3). As the infection front progresses, healthy cells are reached which in their turn activate defenses (encompassing upregulation of defense genes and production of defensive metabolites), which the pathogen attempts to circumvent. This progressing pattern of pathogen recognition - plant defense - plant cell death, continues throughout the plant tissue until the infection progression can be halted or until the pathogen gains the upper hand and the plant tissue is dead. This cycle entails that the transcriptome, proteome and metabolome changes according to the location of the infection front (healthy, newly infected or already invaded tissue).

The spatial differences in plant defenses have already been described in literature. For example, in the tomato – *Botrytis* pathosystem, this has been demonstrated by Seifi *et al.* (2013a). They showed that at the site of infection in the epidermal cells, ROS rapidly accumulated, followed by cell wall fortification. On the other hand, in the mesophyll cells surrounding the infection site, processes aimed to delay pathogen induced senescence by replenishing the TCA cycle through the GABA shunt. This example illustrates that dependent on the type of sampled tissue, different defense strategies (evasion vs. endurance) can be observed.

In our experiments, we inoculated the leaf sheaths with a conidia suspension of *F. graminearum* and excised the leaf sheaths at different time points, for further analysis (RT-

qPCR, metabolomics)(Figure 7-1A). Because of this approach, we were not able to differentiate the defense responses between infected and non-infected tissue. Hence, we need to perform additional experiments to elucidate whether the decrease in glutamine in infected tissue corresponds to an increase in glutamine levels in surrounding healthy tissue. Additionally, fungal N metabolism may also confound results. As previously mentioned, fungal pathogens greatly rely on the host's metabolism to provide the necessary nutrients for its growth and development. Preferred N sources are actually glutamate and glutamine (Bolton & Thomma, 2008). Hence, further research should attempt to delineate whether the effects on primary N metabolism can be attributed to fungal metabolism or whether is primarily driven by plant defense.

7.1.2.4 Glycosylation: mechanism of priming?

The addition of glycosides to metabolites is a well known mechanism in plants to inactivate metabolites and increase their solubility and detoxify xenobiotics. In fact, the accumulation of inactive glycosylated compounds constitutes a possible mechanism of priming (Pastor *et al.*, 2014). Following a priming signal, glycosylated compounds are produced and stored in the vacuole, where upon cellular damage, they are released and deglycolized to convert to their active configuration. Our untargeted metabolomics analysis revealed a significant increase of glycosylated metabolites in response to Z-3-HAC exposure (Chapter 5), whereas in the other treatments these were barely detectable. However, we were not able to further identify the glycosylated compounds without the use of NMR spectroscopy, so at the moment we are not able to discern whether these glycosylated compounds are involved in plant defense.

The group of benzoxazinoids are also a group of glycosylated defensive compounds in plants, which are transported to the vacuole and upon stress are transformed to their active aglucons. We identified the benzoxazinoid DIMBOA in our untargeted analysis, which was significantly lower in primed plants (Figure 5-6).

The lower level of DIMBOA in Z-3-HAC + *Fg* treated seedlings may imply that either less DIMBOA is formed through a downregulation of β-glucosidase or substrate limitation of DIMBOA-Glc or either a higher conversion of DIMBOA to MBOA (6-methoxybenzoxazolin-2-one). In addition, DIMBOA-Glc can be methylated to form HDMBOA-Glc (2-hydroxy-4,7-dimethoxy-1,4-benzoxazin-3-one glucoside), which can also be converted to its aglucon HDMBOA (2-hydroxy-4,7-dimethoxy-1,4-benzoxazin-3-one)(Figure 7-2), which both exert negative effects against invading insects and fungi.

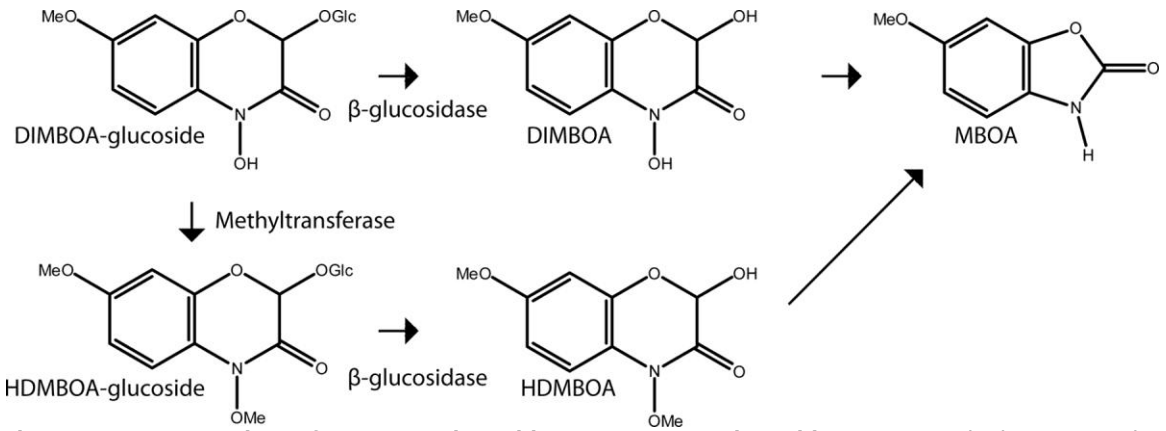


Figure 7-2: Conversion of DIMBOA-glucoside to HDMBOA-glucoside. DMBOA-Glc is converted to its methylated form HDMBOA-Glc by S-adenosyl-L-methionine:DIMBOA-Glc 4-O-methyltransferase. The glycoside compounds are stored in the vacuole and following cellular damage these are converted by β-glucosidases to its aglucon HDMBOA. (Meihls *et al.*, 2012). Abbreviations: **DIMBOA: 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one; HDMBOA: 2-hydroxy-4,7-dimethoxy-1,4-benzoxazin-3-one; MBOA: 6-methoxybenzoxazolin-2-one.**

However, we currently have no further information concerning levels of DIMBOA-Glc, HDMBOA-Glc, HDMBOA and MBOA. It remains to be determined how plants may benefit from a reduction in DIMBOA after exposure to Z-3-HAC. However, a higher conversion of DIMBOA-Glc to HDMBOA-Glc in response to Z-3-HAC may offer an explanation.

7.1.3 JA and SA are involved in the priming response

In Chapter 4, we have shown that wheat defense against *F. graminearum* is achieved by a sequential activation of SA and JA dependent defenses. While the involvement of SA and JA in the defense response was in accordance to the biphasic infection character, we were particularly interested whether Z-3-HAC interacted with either of the signaling pathways. One of the experiments involved the analysis of the expression of defense genes after MeSA and MeJA treatment in both control and primed plants. Data revealed that JA dependent gene expression was enhanced in primed plants, whereas gene expression upon activation of SA defense signaling was lower in primed plants (Figure 4-10). Based on these results we concluded that Z-3-HAC primed JA dependent responses in wheat. However, when we took a closer look to phytohormone levels, we surprisingly found high levels of SA at 1 and 6 hai in the Z-3-HAC, and Z-3-HAC + *Fg* treatment, whereas JA levels were not affected by Z-3-HAC (Figure 5-8). This seemingly contrasts with RT-qPCR data, where we saw no, or only a small upregulation of defense genes in Z-3-HAC-treated leaves (Figure 4-11). We would expect that high levels of SA would also result in the activation of defense genes (Figure 4-10). However, because of the lack of RT-qPCR data at the early time points (1 and 6 hai), it remains possible that we missed the increased defense response (Figure 7-3). This stresses the need to include time series experiments with small enough intervals when studying plant-pathogen interactions in order to minimize drawing wrong conclusions (Figure 7-3).

Additionally, while we sampled leaves 1, 6, 24 and 48 hours after inoculation with *Fg*, the primed treatments have already been exposed to Z-3-HAC 15 hours before, thus for the Z-3-HAC treatment, we sampled 16, 21, 39, and 63 hours after treatment. Several other studies have already shown that GLVs induce an upregulation of the expression of SA biosynthesis genes (Bate & Rothstein, 1998; Arimura *et al.*, 2001; Farag *et al.*, 2005) and JA biosynthesis genes (Bate & Rothstein, 1998; Arimura *et al.*, 2001; Gomi *et al.*, 2003; Farag *et al.*, 2005; Kishimoto *et al.*, 2006b; Engelberth *et al.*, 2007) during the first 6 hours after exposure, thus much earlier than the time points of our RT-qPCR data.

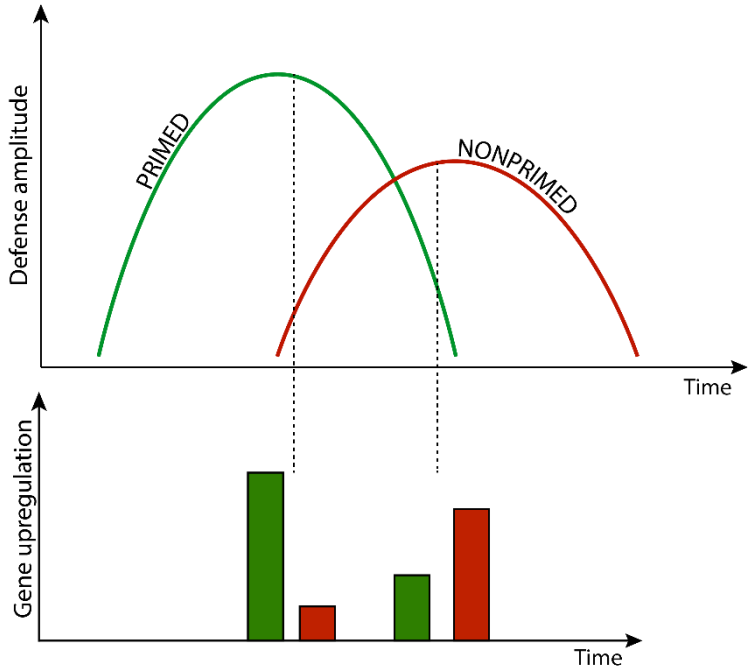


Figure 7-3: Hypothetical model depicting the time course of a primed and nonprimed defense response. The defense response of plants which have been primed are shown in green, nonprimed (native) defense response are shown in red. Analysing gene expression data at *a priori* selected time points entails the risk of wrongly interpreting the data, reaching contrasting conclusions.

In the detached leaf assay, we reported on a clear necrotic front in Z-3-HAC treated leaves, whereas control leaves had more water-soaked lesions (Figure 4-7C). This necrotic front may be an indicator of a HR type response at the front of infection where ROS is produced to induce cell death. As HR type lesions are typical for SA dependent defense (Mur *et al.*, 2008), this advocates in favor of an enhanced SA response. Based on this observation, an early increased SA-dependent defense response thus seems a more likely candidate to clarify the increased defense against *F. graminearum* after Z-3-HAC exposure. Furthermore, the increase in ROS would account for the increase in DON, as ROS is known to induce DON production in *F. graminearum* (Audenaert et al., 2013).

The effect of Z-3-HAC on SA levels and JA dependent defense are not mutually exclusive as GLVs have been associated with both JA and SA responses (Bate & Rothstein, 1998; Kishimoto *et al.*, 2006b; Mirabella *et al.*, 2008; Liu *et al.*, 2012; Tong *et al.*, 2012; Mirabella *et*

al., 2015). As we saw in Chapter 5, pathogen infection adds another layer of complexity to the effect of Z-3-HAC on glutamine levels, where fungal presence is mandatory to induce the drop in glutamine levels. After recognition of a MAMP, signaling pathways may be induced or suppressed in plants to counteract pathogen invasion. This entails that GLV treated plants may display different defense mechanisms, compared to GLV treated plants, which are additionally infected. Hence, to fully understand the effect of GLVs on plant defense, it does not suffice to perform GLV exposure experiments and investigate the effect on defense mechanisms without incorporating MAMPS or pathogens. Furthermore, pathogens possess an array of virulence factors (effectors and toxins, see section 4.2.2), which also interfere with plant defense mechanisms, adding a final level of complexity.

Together these results suggest that Z-3-HAC influences processes which are at the interface of SA and JA signaling. However, without further information on the expression of defense genes at early time points, it cannot be excluded that the observed upregulation is solely dependent on SA signaling.

7.1.4 Priming or induced resistance?

The primed state has been defined by the PRIME-A-PLANT group as: *The physiological condition in which plants are able to better or more rapidly mount defense responses, or both, to biotic or abiotic stress* (Conrath *et al.*, 2006). This entails, in contrast to induced resistance, that plant defense is not or only lightly induced upon priming, but that at the advent of a stress signal, defenses are upregulated much more strongly.

However, it has been recognized by Martinez-Medina *et al.* (2016) that much depends on the type of defense response that is analyzed. By looking at a selection of defensive traits such as PR gene expression, defense metabolites, MPKs, one runs the risk of ignoring the overall defensive state of the plant. To differentiate between induced resistance and priming, Martinez-Medina *et al.* (2016) proposed some key criteria to assess priming: (1) memory, (2) low fitness costs, and (3) more robust defense.

(1) Memory encompasses that after the priming event, the primed state is maintained for a long period of time until the advent of a biotic attack. As mentioned in section 2.3.3, this can be achieved by accumulation of MAPKs, PRRs and epigenetic mechanisms. Unfortunately, we do not have information on these mechanisms, but we found accumulation of glycosylated compounds (Chapter 5). As these compounds remained high at 72 hai, and slowly declined over time, glycosylation processes are a viable option to explain the primed state of the plants. (2) Low fitness costs entail that the overall costs of the priming processes are lower than the costs in case of infection/infestation (Figure 2-4). The accumulation of glycosylated compounds demands that resources are allocated to the production of these compounds and thus encompass a fitness cost as these resources can temporarily not be

used in growth and development processes. However, as can be seen in Figure 5-11, the amount of glycosylated metabolites declines over time, which shows that Z-3-HAC only transiently activated the glycosylation processes. While we do not have hard evidence for fitness costs, we were not able to visually distinguish primed from non primed seedlings in the different treatments. However, long term exposure studies are needed to quantify possible fitness costs.

The third criterion for priming consisted of a (3) more robust defense. In Chapter 4 and 6, we showed for several pathosystems that priming by Z-3-HAC resulted in increased defense, fulfilling this criterion.

In addition, the aforementioned three criteria (memory, low fitness costs and more robust defense) were supplemented with two additional characteristics: (4) broad spectrum activity (encompassing enhancement of all defense responses against (a)biotic stress) and (5) low ecological costs (encompassing minor effects on mutualists or reduced intra-interspecific competitive power). However, we believe that these additional criteria are difficult, if not impossible to fulfill. Because of the many antagonistic signaling pathways in plants, interference or enhancement of one of these pathways, will most likely negatively influence other signaling pathways, thereby overriding these two criteria (Pieterse *et al.*, 2012; De Vleesschauwer *et al.*, 2014; Nguyen *et al.*, 2016). This was also exemplified in our study, where we found that Z-3-HAC enhanced defense against several pathogens, but also found enhanced susceptibility to other pathogens (Chapter 6).

Based on the criteria set by Martinez-Medina *et al.* (2016), we conclude with a high level of confidence that treatment with Z-3-HAC primes defense responses in plants.

7.1.5 GLV signaling, a central role for Z-3-HOL?

The mechanism by which GLVs are perceived and the signal is transduced, remains enigmatic (section 3.5). Sugimoto *et al.* (2014) demonstrated in tomato that exogenous Z-3-HOL is taken up by leaves and transformed to (di)glycosides and thus presents a mode of GLV metabolization. In Chapter 5, we found compelling evidence that hexenylglycosides are also formed after Z-3-HAC exposure (metabolite 3444).

Because of the formation of hexenyl (di)glycosides after Z-3-HOL exposure and Z-3-HAC exposure (after action of esterases), it is tempting to speculate that Z-3-HOL and its (di)glycoside constitute the main signaling compounds in GLV-mediated defense. In the event of GLV release by damaged plants, these are perceived by neighboring plants, which activate priming mechanisms and store the GLVs under the form of hexenyl glycosides. Following a biotic attack, this pool of hexenylglycosides is released and may be converted to its aglucons, activating signaling mechanisms and defense.

As evidence is culminating that GLV signaling also occurs independently of SA and JA (Mirabella *et al.*, 2008; Wei *et al.*, 2011; Mirabella *et al.*, 2015), added to the fact that GLV biosynthesis is closely linked to jasmonates, one may hypothesize that GLVs can be viewed as another class of plant (defense) hormones. While the fast production of GLVs, the effect on plant defense, the multifunctional properties, and the conserved nature within the plant kingdom advocate in favor of a role as (defense) hormone, the effect of GLVs on growth responses still remains ambiguous. We found evidence of fast growth inside the leaf sheath which resulted in plant death (Figure 4-13). However, this effect was only visible at a high concentration of Z-3-HAC. In rice, the *OsHPL3* mutant, with reduced E-2-HAL production, showed differences in growth compared to WT plants. While young plants did not differ, at around 2 months, mutant plants exhibited lesion mimic phenotype trough development of HR like lesions, coinciding with reduced tillers and seed set ratio per plant., which led the authors to conclude that HPL is indispensable for normal growth and development (Liu *et al.*, 2012). However, they reported on enhanced JA production at the appearance of the lesion mimic phenotype in the mutant plants. Hence it cannot be excluded that the phenotype can be attributed to enhanced JA levels. On the other hand, *Arabidopsis* ecotype Col-0 contains a natural truncated non-functional HPL, and produces no detectable GLV amounts (Duan *et al.*, 2005) and displays normal growth and development, which contradicts a role for GLVs as an indispensable plant hormone. The *Arabidopsis* Landsberg erecta ecotype, contains a functional HPL and the *hpl* mutants has been shown to interfere with defense against *Ps. syringae* (Scala *et al.*, 2013b) and *B. cinerea* (Shiojiri *et al.*, 2006a). Hence, GLVs rather seem to play a role as defense hormone, than to constitute a novel classical hormone.

7.1.6 GLVs: a promising agronomic tool?

To strive for a more sustainable agronomy, the use of IPM in agriculture is encouraged (European directive 2009/128/CE). This directive urges towards a decline in pesticide use and or a more effective pesticide management by November 2018. Because of the natural origin of GLVs and its plethora of functions, this group of BVOCs may play an important role in the IPM framework in regard to the monitoring (Stenberg *et al.*, 2015), trapping (Grant *et al.*, 2011; Ryall *et al.*, 2012), disrupting (Ochieng *et al.*, 2002) and repelling of insect pests, or in regard to the attraction of insect parasites or in the improvement of plant disease resistance (Cook *et al.*, 2006; Shrivastava *et al.*, 2010; Pickett & Khan, 2016). However, GLV usage may also entail less desirable effects on plants.

7.1.6.1 GLVs, inducing resistance or susceptibility?

It can be expected that a trade-off occurs when plants are constitutively producing GLVs or when plants are treated with GLVs, leading to the activation of defense pathways. This will

not only result in high maintenance costs but can additionally suppress other defense signaling pathways, leaving them more vulnerable against other insects/pathogens. For example, exogenously applied E-2-HAL in *Arabidopsis* activated JA dependent defenses, which promoted susceptibility to *Pseudomonas syringae* (Scala *et al.*, 2013b). This dual response has also been found in another study in which they overexpressed *CsiHPL1*, the gene coding for HPL, in tomato (Xin *et al.*, 2014). This resulted in enhanced constitutive production of Z-3-HAL and Z-3-HOL and lower JA accumulation. This coincided with a higher resistance against the necrotrophic fungus *Alternaria alternata* f. sp. *lycopersici*. However, susceptibility to larvae of the insect herbivore *Prodenia litura* increased. Thus, GLVs rather than JA contributed to resistance against *A. alternata*.

In Chapter 6, we tested the applicability of Z-3-HAC as a priming agent in different pathosystems of the economically important crops wheat and rice and also found both positive and negative effects (Figure 6-13). Z-3-HAC induced resistance in wheat against *F. graminearum* and *F. poae*, while it made wheat more susceptible to *B. graminis* and *Z. tritici*. Rice became more resistant against *M. oryzae, X. oryzae pv. oryzae, R. solani* and *M. graminicola*, but Z-3-HAC had no effect against *C. miyabeanus* in rice. These contrasting outcomes can probably be traced back to antagonistic signaling pathways which are influenced by GLVs. Because of this antagonistic signaling, a widespread use of GLVs in agronomy entails certain risks (e.g. increased DON production). This illustrates that a deeper understanding of the signaling pathways influenced by GLVs is necessary before blindly employing GLVs in agricultural practices and that the usage of GLVs is probably restricted for certain crops and diseases. However, GLVs may still be used in precision agriculture practices and in regions in which there is a high pressure of a plant disease. Additionally, because of the transient priming effect, GLV application may be timed according to crucial periods for fungal infection so it does not overlap with critical periods for other diseases.

7.1.6.2 From the lab to the field

Many studies on the interactions of GLVs with plant resistance are performed in laboratory conditions. To this day, the transfer of experiments from the lab to the field still remains a bottleneck and poses a lot of challenges (Van Baarlen *et al.*, 2007). The use of GLVs as a biocontrol agent against insects has already been tested in a maize field (von Mérey *et al.*, 2011). Slow release dispensers consisting of a blend of E-2-HAL, Z-3-HAL, Z-3-HOL and Z-3-HAC were used to test its efficacy in controlling insect pests. Counterintuitively, fields which have been treated with GLVs showed a higher abundance of *Spodoptera frugiperda* larvae, whereas parasitism rates were similar between treated and control plots. This could be attributed to the attraction of Coleoptera to GLVs. The use of GLVs has generally been looked at from the perspective of herbivore repellence or attraction of a third trophic level.

Another approach focuses on the plant defense. Recently, results were published of a three year field experiment in which plots of soybean plants were regularly exposed to volatiles of cut goldenrod plants (Shiojiri *et al.*, 2017). The number of seeds harvested did not differ between treated and control plots, so BVOC treatment did not influence seed yields. However, control plots had a significant higher level of leaf and seed damage caused by lepidopteran larvae and stinkbugs, compared to treated plots. Exposure of soybean plants to the BVOCs of cut goldenrod plants (containing both GLVs and terpenoids) resulted in the increase of defensive compounds in soybean plants. The benefits of intercropping in a push-pull strategy has already been shown (Pickett & Khan, 2016). Analogous to this strategy, providing green borders around and between crops, which are cut at regular intervals may provide priming signals to the crops to enhance its defenses at the advent of herbivore or pathogen damage.

When considering GLVs as an agronomic tool, migration speed and the reaction kinetics of GLVs in the atmosphere should not be overlooked. The atmospheric lifetimes of C_6 hexenols are about 1-4 hours through atmospheric reactions with OH and O_3 radicals. Thus, after emission these GLVs will be removed quickly from the atmosphere showing that application of GLVs in agronomical practices will not result in much residues providing a green/fast application (Gai *et al.*, 2015). However, the lack of persistent presence in the atmosphere implies that numerous applications of GLVs over the growing season should be done or that slow release devices should be utilized (Heuskin *et al.*, 2011; Bakry *et al.*, 2016; Pickett & Khan, 2016). This also entails that other risks such as habituation need to be taken into account as deterrent or attractant properties of insects towards GLVs can be overcome with time. Insects are able to associate certain volatiles with great rewards (good food resource) or no rewards (absence of food source). When volatile cues are decoupled from food rewards, insects can habituate to these BVOCs rendering the use of GLVs in herbivore repellence/parasite attraction obsolete (Wang *et al.*, 2008a). To avoid learning behavior by insect predators due to the falseness of the signal, Stenberg *et al.* (2015) proposes to provide food rewards under the form of floral or extraflorar nectar.

These examples highlight the importance of taking into account the interaction of BVOCs with the environment ranging from atmospheric reactions to learning behavior/habituation/repellence of insects and possible negative consequences for plants against other pests/pathogens. More field research is needed to investigate whether the implementation of GLVs in agronomy provides additional protective measures against pests and diseases.

7.2 Future perspectives

7.2.1 Determinants of GLV production

While our meta-analysis revealed some interesting trends (e.g. the high production of GLV after fungal infection), more questions about the determining factors in GLV production arose: Do generalist herbivores and necrotrophic fungi induce GLV emissions differently compared to specialist herbivores and biotrophic fungi? What is the underlying reason for the difference in GLV production between monocots and dicots? What is the ecological relevance of *Z*-3- to *E*-2-conversion of GLVs for plants and insects?

Comparative studies on specialist versus generalist herbivores, necrotrophic versus biotrophic fungi and monocots versus dicots will help to understand how different plants react upon different kind of stress. Additionally, the recent identification of a (3*Z*):(2*E*)-enal isomerase and the engineering and characterization of knock-down or knock-out lines will help to understand the physiological function of this enzyme for plants and insects.

7.2.2 GLVs, in search of the mode of signaling

Despite the progress which has been made the last years in unraveling the functions and mechanisms underlying GLV perception and signaling, the question remains: How do plants perceive GLVs and via which mechanism is the signal transduced?

We strongly believe that this question is central in GLV research and answering it will quickly progress applications in agronomy (e.g. breeding, genetic engineering and IPM).

Transcriptomic studies and forward genetics have been used to make the first steps towards the understanding of the early signaling mechanisms in plants. Such strategies, but also more chemically related approaches are necessary to understand the mechanisms behind GLV perception in plants.

As previously mentioned, the spatiotemporal aspects of plant-pathogen interactions should not be overlooked. Therefore, future experiments should incorporate both timing and location factors by performing analyses at different time points and differentiating between infected and non-infected tissue. The use of real time *in vivo* imaging techniques which can monitor the progression of a pathogen offers exciting opportunities.

7.2.3 Valorization of GLVs

Because of its multifunctionality and high conserved nature throughout the plant kingdom, GLVs are of particular interest for agronomic practices. We tested the applicability of Z-3-HAC in wheat and rice and found promising results for different pathosystems. However,

these experiments were performed under laboratory conditions while transfer to fields imposes a lot of challenges (see 7.1.6.2). Hence, future experiments should additionally focus on the valorization of GLVs in agronomy and following questions need to be addressed: which GLV application devices should be used, are single compound GLV or combinations of GLVs more potent, what are the long term effects of GLV exposure, and what are the threshold levels between growth and primed defense?

In this doctoral thesis, we focused on monocotyledonous crops because of its importance as staple crops. However, GLVs may also act as defense priming agents in dicotyledonous plants. More specifically, plant species which are grown in intensively managed greenhouses (tomato, bellpepper, lettuce, berries and ornamental flowers) may provide interesting opportunities as GLV application can be more precisely controlled. Already, IPM measures are employed in greenhouses by using biocontrol measures such as the introduction of predatory mites and parasitic wasps. Thus, research involving the potential of GLVs in these plant species may further reduce pesticide usages in these plants.

7.2.4 Looking at other plant hormones

In section 2.2, we discussed the importance of plant hormones in orchestrating the defense outcome during plant-pathogen interactions. In this doctoral study, we found evidence for a role of SA (section 5.5.3) and JA (section 4.5.3) in modulating the Z-3-HAC priming response, while no effect of Z-3-HAC on ABA or IAA synthesis was found. However, this does not imply that Z-3-HAC cannot have an effect on processes downstream of these hormones.

From our exposure experiment, we saw at high concentrations of Z-3-HAC, a zone of rapid growth inside the leaf sheath (Figure 4-13), which points to an effect of Z-3-HAC on growth responses. Both IAA and GA play important roles in the growth regulation of plants. GA is of particular interest through its influence on DELLA proteins, which are at the interface of growth and (JA) defense (De Bruyne *et al.*, 2014). DELLAs represent a class of nuclear growth repressing proteins, which inhibit PHYTOCHROME INTERACTING FACTOR (Huot *et al.*, 2014) and which additionally interact with JAZ proteins, thereby relieving the suppression of JA responsive genes. However at high concentrations of GA, DELLAs are targeted for degradation, resulting in the relief of the growth suppression on the one hand and in a continued repression of JA dependent gene activation on the other hand.

Unfortunately, we were not able to detect GA3 in our samples. However, spiked samples with GA3 did only show minor peak areas using HPLC-MS; and the detection limit was above GA3 values which have previously been reported in wheat. Thus, we should employ a different method of GA3 detection and quantification before conclusions can be drawn. Already some preliminary experiments were performed in which application of the GA

inhibitor chlormequat resulted in a loss of priming responses. This suggests that the Z-3-HAC-induced response requires an intact GA pathway. As GA antagonizes JA dependent signaling, and we found high SA levels upon Z-3-HAC exposure, this advocates in favor of a central role of SA in the priming response. To fully disclose the involvement of GA and/or DELLA, more experiments using exogenously applied GA, GA inhibitors and DELLA mutants are mandatory.

7.2.5 Glycosyltransferases in wheat

One of the most surprising effects in plants upon Z-3-HAC exposure is the accumulation of glycosylated components. Identification of the involved GT's is not straightforward as 41 different GT families have been identified in wheat, which are involved in several biochemical processes (Sado *et al.*, 2009). Structural identification of these metabolites using NMR spectroscopy may aid in the identification of the glycosylated metabolites and may limit the number of candidate GTs. Furthermore, identification of the metabolites may provide information on the metabolite pathways that are involved in the priming responses.

Summary

Green Leaf Volatiles (GLVs) are a group of plant volatiles which consist of 6 carbon molecules which derive their name from the typical grassy fragrance. Although GLVs have since long been known to constitute an important part of the volatile spectrum of leaves, they have mostly been associated with wounding responses, insect damage and insect host plant location. It was only later that researchers found that this group of volatiles is able to activate and prime defense responses in plants. Priming constitutes a mechanism in which defenses are prepared so plants respond more strongly at the advent of a future attack. Recently, it has been shown that the production of GLVs is not limited to herbivore infested plants, but is also upregulated upon pathogen infection, pointing to a possible central role that GLVs play in the plant defense upon biotic stress.

This doctoral thesis aimed at uncovering the priming potential of the GLV Z-3-hexenyl acetate (Z-3-HAC) in enhancing plant defense against pathogens. To achieve this, we first focused on wheat and the hemi-biotrophic fungal pathogen *Fusarium graminearum*, which is the causal agent of Fusarium Head Blight.

Our infection assays showed that Z-3-HAC confers enhanced resistance against *F. graminearum*. This was the first time that priming by GLVs has been demonstrated to act against fungal pathogens. In ensuing experiments, we tried to pinpoint which defense mechanism was affected by Z-3-HAC treatment. We found that primed plants showed a higher upregulation of defense genes in MeJA challenged plants, whereas primed plants showed attenuated defense gene expression upon MeSA challenge. Based on these results, we proposed a model in which Z-3-HAC primes wheat by enhancing JA dependent defense signaling. While we found that primed plants exhibited enhanced defense, we also found increased levels of the phytotoxin deoxynivalenol, illustrating that pathogens reacted to the increased defense.

By using targeted approaches one runs the risk of overlooking other important mechanisms which are affected by Z-3-HAC priming. Therefore, we performed an untargeted metabolomics analysis to identify new metabolites that are produced or downregulated in response to priming. Out of 4310 metabolites, we fragmented 15 metabolites that contributed most to the predictability of the model and were putatively identified using the *in-silico* web application MetFrag^{TM}. We found strong evidence for the production of glycosylated metabolites upon Z-3-HAC exposure. One of the known priming mechanisms is the storage of inactive glycosylated defensive metabolites in the vacuole, which are released upon damage and then transformed to their active state. Hence, the production of these glycosylated metabolites seems the likely mode of action of Z-3-HAC induced priming. However, further identification steps of these metabolites are mandatory to discern whether

these are indeed involved in defense mechanisms. One of the glycosylated metabolites is believed to be hexenylglycoside, which would imply that Z-3-HAC is taken up by the plant, reduced to its alcohol and glycosylated subsequently, which suggests that hexenylglycosides and its aglucon Z-3-HOL serve as signaling compounds.

One of the metabolites was accurately identified as the benzoxazinoid DIMBOA, which is known to be involved in the defense against *Fusarium* spp. in grasses. However, because of its lower levels in primed plants, this does not seem to be the causal agent for the increased defense. Further analysis of its glycosylated (DIMBOA-Glc) and methylated form (HDMBOA) are needed to know whether these are higher in primed plants and could serve as an explanation for the higher resistance.

The untargeted metabolomics study was amended with a targeted analysis of some key defensive metabolites and plant defense hormones. Surprisingly, we found high levels of SA at 1 and 6 hours after inoculation in primed plants, whereas JA levels were not affected. Furthermore, primed plants had lower levels of glutamate and glutamine, which point to an early transport of nitrogen away from the infected tissue.

Combining data from our gene expression analysis and our metabolomics study, we propose a model in which Z-3-HAC enhances processes at the interface of SA and JA signaling, mediated by glycosylated compounds. Which, in a first phase, aims at preventing the pathogen to obtain a foothold (by increased SA production) and in the second phase enhances JA dependent gene expression.

The results from aforementioned experiments are promising to employ GLVs as a novel agronomic tool. To test the applicability of GLVs in enhancing defense against different pathogens, we performed infection experiments in wheat and rice against pathogens with different lifestyles and infection mechanisms. While wheat became more resistant against *F. graminearum* and *F. poae*, an adverse effect was observed for *B. graminis* and *Z. tritici* in wheat. Rice became more resistant against *M. oryzae, X. oryzae pv. oryzae, R. solani* and *M. graminicola*, but no effect was found against *C. miyabeanus* in rice.

This highlights the intricate defense signaling mechanisms, between which antagonistic crosstalk exists. Thus enhancing certain defense signaling pathways may counteract other signaling pathways.

In summary, we have shown in this doctoral thesis that Z-3-HAC primes wheat and rice for enhanced defense against an array of pathogens, by interfering with SA and JA dependent defense. However, more research on the signaling pathways is mandatory before GLV can be utilized as a novel biocontrol tool in agronomic practices.

Samenvatting

Groen blad volatielen (Eng. Green Leaf Volatiles, GLVs) zijn een groep plant volatielen die bestaan uit 6 koolstofatomen. GLVs maken een belangrijk deel uit van het spectrum aan volatielen van planten en worden gekenmerkt door de typische geur van vers gemaaid gras. In het verleden werd productie van GLVs door planten vooral geassocieerd met mechanische schade of schade door insecten. Het was pas later dat onderzoekers vaststelden dat GLVs in staat zijn om het defensiemechanisme van de plant aan te schakelen en te primen. Primen van verdediging is een mechanisme waarbij de defensie van de plant wordt voorbereid om sneller en sterker te reageren bij een toekomstige biotische aanval.

Recent is aangetoond dat planten ook GLVs produceren in respons op pathogene schimmels. De productie van GLVs na een aanval van zowel insecten en schimmels duidt al op een centrale rol van GLVs in de verdediging van planten tegen biotische stress.

Deze doctoraatsthesis had als doel het ontrafelen van het primingspotentieel van de GLV Z-3-hexenyl acetaat (Z-3-HAC) om de verdediging van planten te verhogen tegen pathogenen. Om dit doel te bereiken, hebben we eerst gefocust op het model systeem tarwe en de hemi-biotrofe schimmel *Fusarium graminearum*, verantwoordelijk voor de ziekte aarfusarium.

Onze infectie assays toonden aan dat Z-3-HAC verhoogde verdediging induceert tegen F. graminearum. Dit was de eerste keer dat priming door GLVs tegen schimmelinfecties werd aangetoond. In daaropvolgende experimenten trachtten we de mechanismen die betrokken zijn bij de priming te identificeren. Experimenten toonden aan dat geprimede planten een hogere genexpressie vertoonden na behandeling met MeJA, terwijl na behandeling met MeSA genexpressie deels onderdrukt werd. Gebaseerd op deze experimenten stelden we een model voor waarbij priming met Z-3-HAC de JA-afhankelijke verdediging versterkt. Naast de verhoogde verdediging vertoonden geprimede planten ook een verhoogde aanwezigheid van het mycotoxine deoxynivalenol, wat aantoont dat ook plant pathogenen reageren tegen de verhoogde verdediging.

Door een gerichte onderzoeksmethodiek toe te passen loopt men het risico om belangrijke (ongekende) mechanismes die beïnvloed worden door Z-3-HAC priming over het hoofd te zien. Om deze reden hebben we een ongerichte holistische metabolomics analyse uitgevoerd om nieuwe metabolieten te ontdekken die geproduceerd of geïnhibeerd worden na priming. Uit een groep van 4310 metabolieten werden uiteindelijk 15 metabolieten die het meeste bijdragen tot de voorspelbaarheid van het OPLS-DA model gefragmenteerd. Deze werden vervolgens *in-silico* geïdentificeerd met de web applicatie MetFrag™. We vonden hierbij sterke aanwijzingen voor de productie van geglycosyleerde metabolieten na Z-3-HAC blootstellingen. Een van de gekende priming mechanismes is de productie en opslag van

inactieve geglycosyleerde metabolieten die na (biotische) schade omgezet worden naar hun actieve vorm. Dus, de productie van geglycosyleerde metabolieten vormt een mogelijk werkingsmechanisme om priming door Z-3-HAC te verklaren. Echter, verdere identificatie van deze metabolieten is *nodig om te weten of deze betrokken zijn in de plantdefensie.*

Verder hebben we sterke aanwijzingen dat een van de geglycosyleerde metabolieten hexenylglycoside is, wat impliceert dat Z-3-HAC opgenomen wordt door de plant, gereduceerd en vervolgens geglycosyleerd wordt. Dit laat vermoeden dat hexenylglycosides en hun aglucons dienst doen als signaalmolecules.

Een van de 15 metabolieten werd correct geïdentificeerd als de benzoaxizinoide DIMBOA, die ook betrokken is bij de verdediging tegen *Fusarium spp*. in de *Poaceae* familie. Door het lager niveau aan DIMBOA in de geprimede planten lijkt het niet dat DIMBOA aan de oorzaak van de verhoogde resistentie ligt. Onderzoek naar de geglycosyleerde vorm (DIMBOA-Glc) of de gemethyleerde vorm (HDMBOA) is nodig om te ontrafelen of deze inderdaad betrokken zijn bij de verhoogde verdediging.

Naast een ongerichte metabolomics studie, hebben we buiten plantenhormonen ook gericht gekeken naar enkele metabolieten die een belangrijke rol spelen in de plantenverdediging. In tegenstelling tot onze verwachtingen vonden we hogere niveaus van SA 1 en 6 uur na inoculatie in de geprimede planten terwijl JA niveaus niet verschilden van de controle behandeling. Verder vonden we in geprimede en geïnfecteerde planten lagere niveaus van de aminozuren glutamaat en glutamine wat duidt op een vroeg stikstof (N) transport weg van de infectiesite.

Gebaseerd op onze bevindingen van de genexpressie en metaboloomdata, stellen we een model voor waarbij Z-3-HAC processen versterkt die op de kruising zitten van de SA-en JA-afhankelijke signalisering, gemedieerd door geglycosyleerde metabolieten. Welke, in een eerste fase gericht zijn tegen de kolonisatie van een pathogeen (door verhoogde SA productie en bijhorend HR respons), en in een tweede fase JA-afhankelijke genexpressie versterkt.

De resultaten van voorgaande experimenten zijn veelbelovend voor het gebruik van GLVs als een innovatief instrument in de gewasbescherming. Om de toepasbaarheid van Z-3-HAC te testen in de verdediging tegen verschillende pathogenen, hebben we verscheidene infectie-experimenten uitgevoerd met tarwe en rijst als modelgewassen.

We vonden na priming met Z-3-HAC een verhoogde resistentie van tarwe tegen *F. graminearum* en *F. poae*, terwijl we een hogere gevoeligheid vonden voor *B. graminis* and *Z. tritici*. Rijst werd resistenter tegen *M. oryzae, X. oryzae pv. oryzae, R. solani* en *M. graminicola*, maar priming had geen effect tegen *C. miyabeanus*.

Deze resultaten illustreren de complexe signalisatiesmechanismes in de verdediging van de plant en tonen aan dat de versterking/activatie van een signalisatiepathway een antagonistische effect kan hebben op andere signalisatiepathways.

In deze doctoraatsthesis hebben we aangetoond dat Z-3-HAC de verdediging van tarwe en rijst primet tegen verschillende pathogenen door in te spelen op SA- en JA-afhankelijke verdediging. Echter, meer onderzoek naar de signalisering is nodig voordat GLVs toegepast kunnen worden als een nieuw instrument in gewasbescherming.

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Chapter 8 Curriculum Vitae

8.1 Personal Information

Name: Maarten Ameye
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Date of birth: March 31, 1987
Place of birth: Brugge
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8.2 Education

1999–2001: Middle School Spes Nostra, Zedelgem

2001–2005: Secondary school, Sint-Franciscus Xaveriusinstituut, Brugge

2006–2011: Bio-engineer, option: Environmental Technology, Ghent University, Gent
master thesis: Heeft een verhoogde CO_2-concentratie een effect op de gevolgen van hittegolven bij bomen? Promotors: K. Steppe, Robert Teskey

8.3 Scientific output

8.3.1 Publications in international journals (ISI papers)

Ameye M3, Allmann S3, Verwaeren J, Haesaert G, Smagghe G, Schuurink R, Audenaert K (2017) Green leaf volatile production by plants: a meta-analysis. New Phytologist, doi:10.1111/nph.14671

De Zutter, N, Audenaert K, Ameye M, Haesaert G, Smagghe G (2016) Effect of the mycotoxin deoxynivanenol on grain aphid *Sitobion avenae* and its parasitic wasp *Aphidius ervi* through food chain contamination. Arthropod-Plant Interactions. 10: 323 doi:10.1007/s11829-016-9432-1

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Ceuppens B, Ameye M, Van Langenhove H, Roldan-Ruiz I, Smagghe G, (2015) Characterization of volatiles in strawberry varieties ‘Elsanta’ and ‘Sonata’ and their effect on bumblebee flower visiting. Arthropod-Plant interactions 9 (3), 281-287

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Giner M, Avilla J, De Zutter N, Ameye M, Balcells, Smagghe G. (2013). Insecticidal and repellent action of allyl esters against *Acyrthosiphon pisum* (Hemiptera: Aphididae) and *Tribolium castaneum* (Coleoptera: Tenebrionidae). Industrial Crops and Products 47(5): 63-68.

Ameye M, Wertin TM, Bauweraerts I, McGuire MA, Teskey RO, Steppe K. (2012). The effect of induced heat waves on *Pinus taeda* and *Quercus rubra* seedlings in ambient and elevated CO_2 atmospheres. New Phytologist 196(2): 448-461.

8.3.2 Oral presentations and contributions

Ameye M, Van Meulebroek L, Vanhaecke L, Smagghe G, Haesaert G, Audenaert K (2017) Uncovering the priming potential of the Green Leaf Volatile Z-3-HAC in wheat, a metabolomics approach, EFPP-SFP, Dunkirk, France

Ameye M, Audenaert K, Smagghe G, Haesaert G (2016) Green Leaf Volatiles Protect Monocot Crops Against Several Pathogens, Gordon Research Seminar, Ventura Beach, California (USA)

Ameye M, Audenaert K, De Zutter N, Haesaert G, Smagghe G. (2015) Exposure to green leaf volatiles primes wheat against FHB but boosts production of the mycotoxine DON, KNPV working group Fusarium, Utrecht, The Netherlands

Ameye M, Audenaert K, De Zutter N, Haesaert G, Smagghe G. (2015) Exposure to green leaf volatiles primes wheat against FHB but boosts production of the mycotoxine DON, International Crop Protection Symposium, Ghent, Belgium

Ameye M, Audenaert K, De Zutter N, Haesaert G, Smagghe G. (2015) Exposure to green leaf volatiles primes wheat against FHB but boosts production of the mycotoxine DON, European Fusarium Seminar, Martina Franca, Italy

De Zutter N, Audenaert K, Ameye M, Haesaert G, Smagghe G. (2014) Interaction between the fungal pathogen Fusarium graminearum and the grain aphid Sitobion avenae. KNPV working group Fusarium, Utrecht, The Netherlands

Bauweraerts I, Wertin TMW, Ameye M, McGuire MA, Teskey RO, Steppe K (2014) Heat waves have a larger impact on forest productivity than shifts in average temperature. Strategic COST event: A Scientific Roadmap for Projections of Global Change Impacts on Forests, Sarajevo, Bosnia-Herzegovina

[3] Contributed equally

Ameye M, Audenaert K, De Zutter N, Haesaert G, Smagghe G. (2013) The use of green leaf volatiles as additional tool in integrated pest management against Fusarium head blight, KNPV working group Fusarium, Utrecht, The Netherlands

Teskey RO, Bauweraerts I, Wertin TMW, Ameye M, McGuire MA, Steppe K (2013) Effect of repeated heat waves, elevated [CO2] and low water availability on growth of tree seedlings. 98th ESA convention, Minneapolis, Minnesota (USA).

Teskey RO, Bauweraerts I, Wertin TMW, Ameye M, McGuire MA, Steppe K (2012) Impact of heat waves, drought stress and elevated [CO_2] on northern red oak seedlings. 97th ESA convention, Portland, Oregon (USA).

Bauweraerts I, Wertin TMW, Ameye M, McGuire MA, Teskey RO, Steppe K (2012) Impact of heat waves on *Quercus rubra* seedlings. International symposium on Wood Structure in Plant Ecology and Biology; 17-20 April, Naples, Italy.

Bauweraerts I, Wertin TMW, Ameye M, McGuire MA, Teskey RO, Steppe K (2012) Extreme heat waves: can trees keep their cool? Newcomers in Nature and Forest Research; 16 March, Brussels, Belgium.

8.3.3 Poster presentations and contributions

Ameye M, Audenaert K, Smagghe G, Haesaert G (2016) Green Leaf Volatiles (GLVs) Protect Monocot Crops Against Several Pathogens, Gordon Research Conference, Ventura Beach, California (USA)

Ameye M, Audenaert K, De Zutter N, Steppe K., Haesaert G, Smagghe G. (2014) Exposure to green leaf volatiles primes wheat against Fusarium Head Blight, MPMI Congress, Rhodes, Greece

Bauweraerts I, Wertin TMW, Ameye M, McGuire MA, Teskey RO, Steppe K (2011) The effect of heat waves on *Quercus rubra* seedlings. IE awards 2011; 1 December, Antwerp, Belgium

Tutorship master thesis

Tijs Dewulf (2015–2016). Gebruik van GLVs in tarwe. Faculty of Bioscience Engineering, Ghent
University, promotor: Kris Audenaert, tutor: Maarten Ameye.

Jandy Genbrugge (2015–2016). Gebruik van GLVs in rijst. Faculty of Bioscience Engineering, Ghent
University, promotor: Kris Audenaert, tutor: Maarten Ameye.

Dankwoord

1982, zoveel dagen zaten tussen het eerste moment dat ik aan mijn doctoraat begon en het moment dat ik mijn doctoraat voor jullie kan presenteren. Deze periode ging gepaard met vallen en opstaan, momenten dat het even tegenzit, maar die worden overschaduwd door de momenten dat het allemaal meezit. Dit doctoraat is zeker geen individueel werk, maar is tot stand gekomen door verschillende mensen.

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